United States
Environmental Protection
Agency

Office of Pesticides &
Toxic Substances
Washington DC 20460

EPA 560/6-82-002
PB82-232992
August 1982

Toxic Substances

EPA

Environmental Effects Test Guidelines

Part Two

EG-11
August, 1982

ENVIRONMENTAL EFFECTS TEST GUIDELINES

Part Two

FISH EARLY LIFE STAGE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FISH EARLY LIFE STAGE TOXICITY TEST

(a) Purpose. This guideline is intended to be used for assessing the propensity of chemical substances to produce adverse effects to fish during the early stages of their growth and development. This guideline describes the conditions and procedures for the continuous exposure of several representative species to a chemical substance during egg, fry and early juvenile life stages. The Environmental Protection Agency (EPA) will use data from this test in assessing the potential hazard of the test substance to the aquatic environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in Part 792--Good Laboratory Practice Standards, apply here. In addition, the following definitions are applicable to this specific test guideline:

(1) "Acclimation" physiological or behavioral adaptation of organisms to one or more environmental conditions associated with the test method (e.g., temperature, hardness, pH).

(2) "Carrier" solvent or other agent used to dissolve or improve the solubility of the test substance in dilution water.

(3) "Conditioning" exposure of construction materials, test chambers, and testing apparatus to dilution water or to the test

solution prior to the start of the test in order to minimize the sorption of test substance onto the test facilities or the leaching of substances from test facilities into the dilution water or the test solution.

(4) "Control" an exposure of test organisms to dilution water only or dilution water containing the test solvent or carrier (no toxic agent is intentionally or inadvertently added).

(5) "Dilution water" the water used to produce the flow-through conditions of the test to which the test substance is added and to which the test species is exposed.

(6) "Early life stage toxicity test" a test to determine the minimum concentration of a substance which produces a statistically significant observable effect on hatching, survival, development and/or growth of a fish species continuously exposed during the period of their early development.

(7) "Embryo cup" a small glass jar or similar container with a screened bottom in which the embryos of some species (i.e., minnow) are placed during the incubation period and which is normally oscillated to ensure a flow of water through the cup.

(8) "Flow through" refers to the continuous or very frequent passage of fresh test solution through a test chamber with no

recycling.

(9) "Hardness" the total concentration of the calcium and magnesium ions in water expressed as calcium carbonate (mg $CaCO_3$/liter).

(10) "Loading" the ratio of biomass (grams of fish, wet weight) to the volume (liters) of test solution passing through the test chamber during a specific interval (normally a 24-hr. period).

(11) "No observed effect concentration (NOEC)" the highest tested concentration in an acceptable early life stage test: (a) which did not cause the occurrance of any specified adverse effect (statistically different from the control at the 95% level); and (b) below which no tested concentration caused such an occurrence.

(12) "Observed effect concentration (OEC)" the lowest tested concentration in an acceptable early life stage test: (a) which caused the occurrence of any specified adverse effect (statistically different from the control at the 95% level); and (b) above which all tested concentrations caused such an occurrence.

(13) "Replicate" two or more duplicate tests, samples, organisms, concentrations, or exposure chambers.

(14) "Stock solution" the source of the test solution prepared by dissolving the test substance in dilution water or a carrier which is then added to dilution water at a specified, selected concentration by means of the test substance delivery system.

(15) "Test chamber" the individual containers in which test organisms are maintained during exposure to test solution.

(16) "Test solution" dilution water with a test substance dissolved or suspended in it.

(17) "Test substance" the specific form of a chemical substance or mixture that is used to develop data.

(c) Test Procedures--(1) Summary of test. (i) The early life stage toxicity test with fish involves exposure of newly fertilized embryos to various concentrations of a test substance. Exposure continues for 28 days post hatch for the minnows and 60 days post hatch for the trout species. During this time various observations and measurements are made in a specific manner and schedule in order to determine the lowest effect and highest no-effect concentrations of the test substance.

(ii) A minimum of five exposure (treatment) concentrations of a test substance and one control are required to conduct an early life stage toxicity test. The concentration of the test

substance in each treatment is usually 50% of that in the next higher treatment level.

(iii) For each exposure concentration of the test substance and for each control (i.e., regular control and carrier control if required) there should be:

(A) At least two replicate test chambers, each containing one or more embryo incubation trays or cups; and there should be no water connections between the replicate test chambers;

(B) At least 60 embryos divided equally, through randomization, between the embryo incubation trays or cups for each test concentration and control (i.e., 30 per embryo cup with 2 replicates);

(C) All surviving larvae divided equally between the test chambers for each test concentration and control (e.g., 30 larvae per test chamber with 2 replicates).

(iv) Duration. (A) For fathead minnow and sheepshead minnow a test begins when the newly fertilized minnow embryos (less than 48-hours old) are placed in the embryo cups and are exposed to the test solution concentrations. The test terminates following 28 days of post-hatch exposure, i.e., 28 days after the newly hatched fry are transferred from the embryo cups into the test chambers.

(B) For brook trout and rainbow trout a test begins when newly fertilized trout embryos (less than 96-hours old) are placed in the embryo trays or cups and are exposed to the test solution concentrations. The test terminates following 60 days of post-hatch exposure (for an approximate total exposure period of 90 days).

(2) [Reserved]

(3) Range-finding test. (i) A range finding test is normally performed with the test substance to determine the test concentrations to be used in the early life stage toxicity test, especially when the toxicity is unknown. It is recommended that the test substance concentrations be selected based on information gained from a 4- to 10-day flow-through toxicity test with juveniles of the selected test species.

(ii) The highest concentration selected for the early life stage toxicity test should approximate the lowest concentration indicated in any previous testing to cause a significant reduction in survival. The range of concentrations selected is expected to include both observed effect and no-observed effect levels. The dilution factor between concentrations is normally 0.50, however, other dilution factors may be used as necessary.

(4) Definitive test--(i) General. (A) A test should not be initiated until after the test conditions have been met and the

test substance delivery system has been observed functioning properly for 48-hours. This includes temperature stability, flow requirements of dilution water, lighting requirements, and the function of strainers and air traps included in the water-supply system, and other conditions as specified previously.

(B) New holding and testing facilities should be tested with sensitive organisms (i.e., juvenile test species or daphnids) before use to assure that the facilities or substances possibly leaching from the equipment will not adversely affect the test organisms during an actual test.

(C) Embryos should be acclimated for as long as practical to the test temperature and dilution water prior to the initiation of the test.

(D) When embryos are received from an outside culture source (i.e. rainbow and brook trout) at a temperature at variance with the recommended test temperature they should be acclimated to the test temperature. When eggs are received, they should be immediately unpacked and the temperature of the surrounding water determined. Sudden temperature changes should be avoided. Acclimation to the appropriate test temperature should be accomplished within a period of six hours, and should incorporate the use of dilution water.

(E) Embryos should be visually inspected prior to placement in the embryo cups or screen trays. All dead embryos should be discarded. Dead embryos can be discerned by a change in coloration from that of living embryos (e.g. trout embryos turn white when dead). During visual inspection, empty shells, opaque embryos and embryos with fungus or partial shells attached should be removed and discarded. If less than 50 percent of the eggs to be used appear to be healthy, all embryos in such a lot should be discarded.

(ii) Embryo incubation procedures. (A) Embryos can be distributed to the embryo cups or screen trays using a pipette with a large bore or a similar apparatus. Trout embryos can be distributed by using a small container which has been precalibrated to determine the approximate number of embryos it can hold; embryos are measured volumetrically in this manner, and are then poured onto the screen tray (or embryo cup). Trout embryos should be separated on the screen tray so that they are not in contact with each other. A final count will ensure the actual number on the screen tray. After random assignmment, the screen trays or embryo cups are placed in the test chambers.

(B) Each day until hatch the embryos are visually examined. Minnow embryos may be examined with the aid of a magnifying viewer. Trout embryos should not be touched. Trout embryos

should be maintained in low intensity light or in darkness until one-week post hatch, and are usually examined with the aid of a flashlight or under low intensity light. Dead embryos should be removed and discarded. Live embryos which are heavily infected with fungus should be discarded, but should be subtracted from the initial number of embryos used as a basis for the calculations of percentage hatch.

(C) When embryos begin to hatch they should not be handled.

(iii) Initiation of fry exposure. (A) Forty-eight hours after the first hatch in each treatment level, or when hatching is completed, the live young fish should be counted and transferred from each embryo cup into the appropriate test chamber. All of the normal and abnormal fry should be gently released into the test chamber by allowing the fry to swim out of each embryo cup; nets should not be used. The trout embryos incubated on screen trays will hatch out in the test chambers, therefore handling of fish is not necessary.

(B) If necessary, fry can be transferred from one replicate embryo cup to the other replicate within a test concentration to achieve equal numbers in each replicate chamber.

(C) The number of live fry, live normal fry, live embryos, dead embryos and unaccounted for embryos for each cup should be recorded when hatching is deemed complete. Those fry which are

visibly (without the use of a dissecting scope or magnifying viewer) lethargic or grossly abnormal (either in swimming behavior or physical appearance) should be counted. Late hatching embryos should be left in the embryo cups to determine if they will eventually hatch or not. The range of time-to-hatch (to the nearest day) for each cup should be recorded.

(iv) Time to first feeding. (A) The first feeding for the fathead and sheepshead minnow fry should begin shortly after transfer of the fry from the embryo cups to the test chambers. Trout species initiate feeding at swim-up. The trout fry should be fed trout starter mash three times a day ad libitum, with excess food siphoned off daily. The minnow fry should be fed live newly-hatched brine shrimp nauplii (Artemia salina) at least three times a day.

(B) For the first seven days, feeding should be done at minimum intervals of four hours (i.e., 8 am, 12 noon, and 4 pm); thereafter the fry should be fed as indicated below.

(v) Feeding. (A) The fathead and sheepshead minnow fry should be fed newly-hatched brine shrimp nauplii for the duration of the test at approximately 4-hour intervals three times a day during the week and twice on the weekend after the first week. Trout fry should be fed at similar intervals, and may receive live brine shrimp nauplii in addition to the trout starter food

after the first week.

(B) An identical amount of food should be provided to each chamber. Fish should be fed ad libitum for 30 minutes with excess food siphoned off the bottom once daily if necessary.

(C) Fish should not be fed for the last 24 hours prior to termination of the test.

(vi) Carriers. Water should be used in making up the test stock solutions. If carriers other than water are absolutely necessary, the amount used should be the minimum necessary to achieve solution of the test substance. Triethylene glycol and dimethyl formamide are preferred, but ethanol and acetone can be used if necessary. Carrier concentrations selected should be kept constant at all treatment levels.

(vii) Controls. Every test requires a control that consists of the same dilution water, conditions, procedures, and test organisms from the same group used in the other test chambers, except that none of the test substance is added. If a carrier (solvent) is used, a separate carrier control is required in addition to the regular control. The carrier control should be identical to the regular control except that the highest amount of carrier present in any treatment is added to this control. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless

an extra amount is used to prepare the stock solution.

(viii) Randomization. The location of all test chambers and species within the test system should be randomized. A representative sample of the test embryos should be impartially distributed by adding to each cup or screen tray no more than 20% of the number of embryos to be placed in each cup or screen tray and repeating the process until each cup or screen tray contains the specified number of embryos. Alternatively, the embryos can be assigned by random assignment of a small group (e.g., 1-5) of embryos to each embryo cup or screen tray, followed by random assignment of a second group of equal number to each cup or tray, which is continued until the appropriate number of embryos are contained in each embryo cup or screen tray. The method of randomization used should be reported in detail.

(ix) Observations. During the embryo exposure period observations should be made to check for mortality. During the exposure period of the fry, observations should be made to check for mortality and to note the physical appearance and behavior of the young fish. The biological responses are used in combination with physical and chemical data in evaluating the overall lethal and sublethal effects of the test substance. Additional information on the specific methodology for the data obtained during the test procedure are discussed in the following

sections.

(x) Biological data. (A) Death of embryos should be recorded daily and dead embryos removed when discovered to prevent the spread of fungal contamination.

(B) When hatching commences, daily records of the number of embryos remaining in each embryo cup are required. This information is necessary to quantify the hatching success. A record of all deformed larvae should be kept throughout the entire post-hatch exposure. Time to swim-up should be recorded for the trout. Upon transfer of fry from the embryo cups to the test chambers, daily counts of the number of live fish should be made. At a minimum, live fish should be counted on days 4, 11, 18, 25 and (weekly thereafter for the trout species) finally on termination of the test.

(C) The criteria for death of young fish is usually immobility, especially absence of respiratory movement, and lack of reaction to gentle prodding. Deaths should be recorded daily and dead fish removed when discovered.

(D) Daily and at termination of the test, the number of fish that appear (without the use of a magnifying viewer) to be abnormal in behavior (e.g., swimming erratic or uncoordinated, obviously lethargic, hyperventilating, or over excited, etc.) or in physical appearance (e.g., hemorrhaging, producing excessive

mucous, or are discolored, deformed, etc.) should be recorded and reported in detail.

(E) All physical abnormalities (e.g., stunted bodies, scoliosis, etc.) should be photographed and the deformed fish which die, or are sacrificed at the termination of the test, should be preserved for possible future pathological examination.

(F) At termination, all surviving fish should be measured for growth. Standard length measurements should be made directly with a caliper, but may be measured photographically. Measurements should be made to the nearest millimeter (0.1mm is desirable). Weight measurements should also be made for each fish alive at termination (wet, blotted dry and to the nearest 0.01g for the minnows and 0.1g for the trout). If the fish exposed to the toxicant appear to be edematous compared to control fish, determination of dry, rather than wet, weight is recommended.

(G) Special physiological, biochemical and histological investigations on embryos, fry, and juveniles may be deemed appropriate and should be performed on a case by case basis.

(5) Test results. (i) Data from toxicity tests are usually either continuous (e.g. length or weight measurements) or dichotomous (e.g. number hatching or surviving) in nature. Several methods are available and acceptable for statistical

anaylsis of data derived from early life stage toxicity tests; however, the actual statistical methodology to analyze and interpret the test results should be reported in detail.

(ii) The significance level for all statistical testing should be a minimum of P=0.05 (95 percent confidence level).

(A) Example of statistical analysis. (1) Mortality data for the embryonic stage, fry stage and for both stages in replicate exposure chambers should first be analyzed using a two-way analysis of variance (ANOVA) with interaction model. This analysis will determine if replicates are significantly different from each other. If a significant difference between replicates or a significant interaction exists, cause for the difference should be determined. Modification should then be made in the test apparatus or in handling procedures for future toxicity tests. Further calculations should incorporate the separation of replicates. If no significant difference is observed, replicates may be pooled in further analyses.

(2) After consideration of replicate responses, mortality data should then be subjected to one-way ANOVA. The purpose of this analysis is to determine if a significant difference exists in the percentage mortality between control fish and those exposed to the test material.

(3) If the one-way ANOVA results in a F ratio that is significant, it would be acceptable to perform t-tests on the control versus each concentration. A second technique is to identify treatment means that are significantly different; this method should involve the additional assumption that the true mean response decreases generally with increasing concentration. The researcher may also be interested in determining significant differences between concentrations.

(4) Growth data should also be analyzed by one-way ANOVA with the inclusion of a covariate to account for possible differences in growth of surviving fry in embryo cup(s) that contain fewer individuals. This condition can occur in cases when the same amount of food is given to each test chamber regardless of the number of survivors.

(B) Test data to be analyzed. Data to be statistically analyzed are:

(1) Percentage of healthy, fertile embryos at 40-48 hours after initiation of the test. Percentage is based upon initial number used.

(2) Percentage of embryos that produce live fry for release into test chambers. Percentage is based on number of embryos remaining after thinning.

(3) Percentage of embryos that produce live, normal fry for release into test chambers. Percentage is based upon number of embryos remaining after thinning.

(4) Percentage of fry survival at swim-up for trout. Percentage is based upon number of embryos remaining after thinning.

(5) Percentage of embryos that produce live fish at end of test. Percentage is based upon number of embryos remaining after thinning.

(6) Percentage of embryos that produce live, normal fish at end of test. Percentage is based upon number of embryos remaining after thinning.

(7) Weights and lengths of individual fish alive at the end of the test

(C) It is important that fish length and weight measurements be associated with individual test chambers since the density of the fish and available food should be considered in the growth of the organism.

(iii) Acceptability criteria. (A) An early life stage toxicity test is not acceptable unless at least one of the following criteria is significantly different (p=0.05) from control organisms when compared with treated organisms, and the responses are concentration-dependent: mortality of embryos,

hatching success, mortality of fry (at swim-up for trout), total mortality throughout the test, and growth (i.e. weight). If no significant effects occur, but the concentrations tested were the highest possible due to solubility or other physio-chemical limitations, the data will be considered for acceptance.

(B) In addition to obtaining significant effects on the exposed test species, a measure of acceptablity in the response of control fish is also required.

(C) A test is not acceptable if the average survival of the control fish at the end of the test is less than 80 percent or if survival in any one control chamber is less than 70 percent.

(D) If a carrier is used, the criteria for effect (mortality of embryos and fry, growth, etc.) used in the comparison of control and exposed test organisms should also be applied to the control and control with carrier chambers. For the test to be considered acceptable, no significant difference should exist between these criteria.

(E) A test is not acceptable if the relative standard deviation (RSD=100 times the standard deviation divided by the mean) of the weights of the fish that were alive at the end of the test in any control test chamber is greater than 40 percent.

(6) Analytical measurements--(i) Analysis of water quality. Measurement of certain dilution water quality

parameters should be performed every 6 months, to determine the consistency of the dilution water quality. In addition, if data in 30 day increments are not available to show that freshwater dilution water is constant, measurements of hardness, alkalinity, pH, acidity, conductivity, TOC or COD and particulate matter should be conducted once a week in the control and once a week in the highest test substance concentration. Measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable.

(ii) Dissolved oxygen measurement. The dissolved oxygen concentration should be measured in each test chamber at the beginning of the test and at least once daily thereafter (as long as live organisms are present) in one replicate of the control and the high, medium, and low test substance concentrations.

(iii) Temperature measurement. Temperatures should be recorded in all test chambers at the beginning of the test, once weekly thereafter and at least hourly in one test chamber. When possible, the hourly measurement should be alternated between test chambers and between replicates.

(iv) Test substance measurement. (A) Prior to the addition of the test substance to the dilution.water, it is recommended that the test substance stock solution be analyzed to verify the concentration. After addition of the test substance, the

concentration of test substance should be measured at the beginning of the test in each test concentration (including both replicates) and control(s), and in one replicate at each test concentration at least once a week thereafter. Replicates should be alternated each week. If a malfunction in the delivery system is discovered, water samples should be taken from the affected test chambers immediately and analyzed.

(B) The measured concentration of test substance in any chamber should be no more than 30% higher or lower than the concentration calculated from the composition of the stock solution and the calibration of the test substance delivery system. If the difference is more than 30%, the concentration of test substance in the solution flowing into the exposure chamber (influent) should be analyzed. These results will indicate whether the problem is in the stock solution, the test substance delivery system or in the test chamber. Measurement of degradation products of the test substance is recommended if a reduction of the test substance concentration occurs in the test chamber.

(v) Sampling and analysis methodology. (A) Generally, total test substance measurements are sufficient; however, the chemical characteristics of the test substance may require both dissolved and suspended test substance measurements.

(B) For measurement of dissolved or suspended test substance or both, water samples should be taken midway between the top, bottom, and sides of the test chamber and should not include any surface scum or material stirred up from the bottom or sides. For measurement of total test substance, a large volume of the solution in the test chamber should be collected and used as the sample. Samples of test solutions should be handled and stored appropriately to minimize loss of test substance by microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(C) Chemical and physical analyses should be performed using standardized methods whenever possible. The analytical method used to measure the concentration of the test substance in the test solution should be validated before the beginning of the test. At a minimum, a measure of the accuracy of the method should be obtained, on each of two separate days by using the method of known additions, and using dilution water from a tank containing test organisms. Three samples should be analyzed at the next to lowest test substance concentration. It is also desirable to study the accuracy and precision of the analytical method for test guideline determination by use of reference (split) samples, or interlaboratory studies, and by comparsion with alternative, reference or corroborative methods of analysis.

(D) An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. In general, atomic absorption spectrophotometric methods for metals and gas chromatographic methods for organic compounds are preferable to colorimetric methods.

(E) In addition to analyzing samples of test solution, at least one reagent blank also should be analyzed when a reagent is used in the analysis. Also, at least one sample for the method of known additions should be prepared by adding test substance at the concentration used in the toxicity test.

(d) Test conditions--(1) Test species. (i) One or more of the recommended test species will be specified in rules under Part 799 requiring testing of specific chemicals. The recommended test species are:

(A) Fathead minnow (Pimephales promelas Rafinesque).

(B) Sheepshead minnow (Cyprinodon variegatus).

(C) Brook trout (Salvelinus fontinalis)..

(D) Rainbow trout (Salmo gairdneri).

(ii) Embryos used to initiate the early life stage test should be less than 48-hours old for the fathead and sheepshead

minnows, and less than 96-hours old for the brook trout and rainbow trout. In addition, the following requirements should be met:

(A) All embryos used in the test should be from the same source. Embryos should be obtained from a stock cultured in-house when possible, and maintained under the same parameters as specified for the test conditions. When it is necessary to obtain embryos from an external source, caution should be exercised to ensure embryo viability and to minimize the possibility of fungal growth. A description of the brood stock history or embryo source should be made available to EPA upon request.

(B) Test species should be cared for and handled properly in order to avoid unnecessary stress. To maintain test species in good condition and to maximize growth, crowding should be prevented, and the dissolved oxygen level should be maintained near saturation.

(C) Embryos and fish should be handled as little as possible. Embryos should be counted and periodically inspected until hatching begins. When larvae begin to hatch, they should not be handled. Transfer of minnow larvae from embryo cups to test chambers should not involve the use of nets. No handling is necessary following introduction into the test chambers until

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termination of the test.

(D) If fathead minnow embryos are obtained from in-house culture units, the embryos should be gently removed from the spawning substrate. The method for separating the fertilized eggs from the substrate is important and can affect the viability of the embryos; therefore the finger-rolling procedure is recommended.

(E) Disease treatment. Chemical treatments to cure or prevent diseases should not be used before, and should not be used during a test. All prior treatments of brood stock should be reported in detail. Severely diseased organisms should be destroyed.

(2) Test facilities--(i) Construction materials. Construction materials and equipment that contact stock solutions, test solutions, or dilution water into which test embryos or fish are placed should not contain any substances that can be leached or dissolved into aqueous solutions in quantities that can affect test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals from dilution water. Glass, #316 stainless steel, nylon screen and perfluorocarbon plastic (e.g., Teflon®) are acceptable materials. Concrete or rigid (unplasticized) plastic may be used for holding and acclimation

tanks, and for water supply systems, but they should be thoroughly conditioned before use. If cast iron pipe is used in freshwater supply systems, colloidal iron may leach into the dilution water and strainers should be used to remove rust particles. Natural rubber, copper, brass, galvanized metal, epoxy glues, and flexible tubing should not come in contact with dilution water, stock solutions, or test solutions.

(ii) Test chambers (exposure chambers). (A) Stainless steel test chambers should be welded or glued with silicone adhesive, and not soldered. Glass should be fused or bonded using clear silicone adhesive. Epoxy glues are not recommended, but if used ample curing time should be allowed prior to use. As little adhesive as possible should be in contact with the water.

(B) Many different sizes of test chambers have been used successfully. The size, shape and depth of the test chamber is acceptable if the specified flow rate and loading requirements can be achieved.

(C) The actual arrangement of the test chambers can be important to the statistical analysis of the test data. Test chambers can be arranged totally on one level (tier) side by side, or on two levels with each level having one of the replicate test substance concentrations or controls. Regardless of the arrangement, it should be reported in detail and considered in the data analysis.

(iii) Embryo incubation apparatus. (A) recommended embryo incubation apparatus include embryo cups for the minnow species and screen trays for the trout species, although embryo cups can be used for the trout species. Embryo cups are normally constructed from approximately 4-5 cm inside diameter, 7-8 cm high, glass jars with the end cut off or similar sized sections of polyethylene tubing. One end of the jar or tubing is covered with stainless steel or nylon screen (approximately 40 meshes per inch is recommended). The embryo cups should be appropriately labeled and then suspended in the test chamber in such a manner as to ensure that the test solution regularly flows through the cup and that the embryos are always submerged but are not agitated too vigorously. Cups may be oscillated by a rocker arm apparatus with a low rpm motor (e.g., 2 rpm) to maintain the required flow of test water. The vertical-travel distance of the rocker arm apparatus during oscillation is normally 2.5 - 4.0cm. The water level in the test chambers may also be varied by means of a self-starting siphon in order to ensure exchange of water in the embryo cups.

(B) The trout embryo incubation trays can be made from stainless steel screen (or other acceptable material such as plastic) of about 3-4 mm mesh. The screen tray should be supported above the bottom of the test chamber by two folds

of screen or other devices which function as legs or supports. The edges of the screen tray should be turned-up to prevent bump spills and to prevent the embryos from rolling off in the event of excessive turbulence. Suspending or supporting the screen tray off the bottom ensures adequate water circulation around the embryos and avoids contact of embryos with possible bottom debris.

(iv) Test substance delivery system. (A) The choice of a specific delivery system depends upon the specific properties and requirements of the test substance. The apparatus used should accurately and precisely deliver the appropriate amount of stock solution and dilution water to the test chambers. The system selected should be calibrated before each test. Calibration includes determining the flow rate through each chamber, and the proportion of stock solution to dilution water delivered to each chamber. The general operation of the test substance delivery system should be checked twice daily for normal operation throughout the test. A minimum of five test substance concentrations and one control should be used for each test.

(B) The proportional diluter and modified proportional diluter systems and metering pump systems have proven suitable and have received extensive use.

(C) Mixing chambers should be used between the diluter and the test chamber(s). This may be a small container or flow-splitting chamber to promote mixing of test substance stock solution and dilution water, and is positioned between the diluter and the test chambers for each concentration. If a proportional diluter is used, separate delivery tubes should run from the flow-splitting chamber to each replicate test chamber. Daily checks on this latter system should be made.

(v) Other equipment required. (A) An apparatus for removing undesirable organisms, particulate matter and air bubbles.

(B) An apparatus for aerating water.

(C) A suitable magnifying viewer for examination of minnow embryos.

(D) A suitable apparatus for the precise measurement of growth of the fish, including both length (e.g., with metric or ruler caliper or photographic equipment) and weight.

(E) Facilities for providing a continuous supply of live brine shrimp nauplii (Artemia salina).

(F) Facilities (or access to facilities) for performing the required water chemistry analyses.

(vi) Cleaning of equipment. (A) Test substance delivery systems and test chambers should be cleaned before use. Test chambers should be cleaned during the test as needed to maintain

the dissolved oxygen concentration, and to prevent clogging of the embryo cup screens and narrow flow passages.

(B) Debris can be removed with a rubber bulb and large pipette or by siphoning with a glass tube attached to a flexible hose. Debris should be run into a bucket light enough to observe that no live fish are accidentally discarded.

(vii) Dilution water--(A) General. (1) A constant supply of acceptable dilution water should be available for use throughout the test. Dilution water should be of a minimum quality such that the test species selected will survive in it for the duration of testing without showing signs of stress (e.g., loss of pigmentation, disorientation, poor response to external stimuli, excessive mucous secretion, lethargy, lack of feeding or other unusual behavior). A better criterion for an acceptable dilution water for tests on early life stages should be such that the species selected for testing will survive, grow and reproduce satisfactorily in it.

(2) The concentration of dissolved oxygen in the dilution water (fresh or salt) should be between 90% and 100% saturation. When necessary, dilution water should be aerated by means of airstones, surface aerators, or screen tubes before the introduction of the test substance.

(3) Water that is contaminated with undesirable microoganisms (e.g. fish pathogens) should not be used. If such contamination is suspected, the water should be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter before use. Efficacy of the sterilizer can be determined by using standard plate count methods.

(B) Freshwater. (1) Natural water (clean surface or ground water) is preferred, however, dechlorinated tap water may be used as a last resort. Reconstituted freshwater is not recommended as a practical dilution water for the early life stage toxicity test because of the large volume of water required.

(2) Particulate and dissolved substance concentrations should be measured at least twice a year and should meet the following specifications:

Substance	Concentration Maximum
Particulate matter	< 20 mg/liter
Total organic carbon (TOC)	< 2 mg/iter
Chemical oxygen demand (COD)	< 5 mg/liter
Un-ionized ammonia	< 1 ug/liter
Residual chlorine	< 1 ug/liter
Total organoposphorus pesticides	< 50 ng/liter
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs)	< 50 ng/liter
Total organic chlorine	< 25 ng/liter

(3) During any one month, freshwater dilution water should not vary more than 10% from the respective monthly averages of hardness, alkalinity and specific conductance; the monthly pH range should be less than 0.4 pH units.

(C) Saltwater. (1) Marine dilution water is considered to be of constant quality if the minimum salinity is greater than 15 o/oo and the weekly range of the salinity is less than 15 o/oo. The monthly range of pH should be less than 0.8 pH units. Saltwater should be filtered to remove larval predators. A pore size of $\leq$ 20 micrometers (um) is recommended.

(2) Artificial sea salts may be added to natural seawater during periods of low salinity to maintain salinity above 15 o/oo.

(3) Test parameters (i) Dissolved oxygen concentration. It is recommended that the dissolved oxygen concentration be maintained between 90 and 100 percent saturation; but it should be no less than 75 percent saturation at all times for both minnow species and between 90 and 100 percent saturation for the trout species in all test chambers. Dilution water in the head box may be aerated, but the test solution itself should not be aerated.

(ii) Loading and flow rate. (A) The loading in test chambers should not exceed 0.1 grams of fish per liter of test

solution passing through the test chamber in 24 hours. The flow rate to each chamber should be a minimum of 6 tank volumes per 24 hours. During a test, the flow rates should not vary more than 10 percent from any one test chamber to any other.

(B) A lower loading or higher flow rate or both should be used if necessary to meet the following three criteria at all times during the test in each chamber containing live test organisms: (1) the concentration of dissolved oxygen should not fall below 75 percent saturation for the fathead and sheepshead minnows and 90 percent for the rainbow and brook trout; (2) the concentration of un-ionized ammonia should not exceed 1 ug/liter; and (3) the concentration of toxicant should not be lowered (i.e., caused by uptake by the test organisms and/or materials on the sides and bottoms of the chambers) more than 20 percent of the mean measured concentration.

(iii) Temperature. (A) The recommended test temperatures are:

(1) Fathead minnow_____25°C for all life stages.

(2) Sheepshead minnow_____30°C for all life stages.

(3) Rainbow and brook trout_____10°C for embryos.

12°C for fry and alevins.

(B) The actual test temperature during the duration of the test should remain within 1.5°C of the selected test

temperature. It is recommended that the test system be equipped with an automatic alarm system to alert staff of instantaneous temperature changes in excess of 2°C. If the water is heated (i.e., for minnow species), precautions should be taken to ensure that supersaturation of dissolved gases is avoided. Temperatures should be recorded in all test chambers at the beginning of the test and weekly thereafter. The temperature should be recorded at least hourly in one test chamber throughout the test.

(xi) Light. (A) Brook and rainbow trout embryos should be maintained in darkness or very low light intensity through one week post-hatch, at which time a 14-hour light and 10-hour dark photoperiod should be provided.

(B) For fathead and sheepshead minnows, a 16-hour light and 8-hour dark (or 12:12) photoperiod should be used throughout the test period.

(C) A 15-minute to 30-minute transition period between light and dark is optional.

(D) Light intensities ranging from 30 to 100 lumens at the water surface should be provided; the intensity selected should be duplicated as closely as possible for all test chambers.

(e) Reporting. A report of the results of an early life stage toxicity test should include the following:

(1) Name of test, sponsor, investigator, laboratory, and dates of test duration.

(2) Detailed description of the test substance including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and any carriers (solvents) or other additives used.

(3) The source of the dilution water, its chemical characteristics, and a description of any pretreatment.

(4) Detailed information about the test organisms including scientific name and how verified and source history, observed diseases, treatments, acclimation procedure, and concentration of any contaminants and the method of measurement.

(5) A description of the experimental design and the test chambers, the depth and volume of the solution in the chambers, the way the test was begun, the number of organisms per treatment, the number of replicates, the loading, the lighting, a description of the test substance delivery system, and the flow rate as volume additions per 24 hours.

(6) Detailed information on feeding of fish during the toxicity test, including type of food used, its source, feeding frequency and results of analysis (i.e., concentrations) for contaminants.

(7) Number of embryos hatched, number of healthy embryos, time to hatch, mortality of embryos and fry, measurements of growth (weight and length), incidence of pathological or histological effects and observations of other effects or clinical signs, number of healthy fish at end of test.

(8) Number of organisms that died or showed an effect in the control and the results of analysis for concentration(s) of any contaminant in the control(s) should mortality occur.

(9) Methods used for, and the results of (with standard deviation), all chemical analyses of water quality and test substance concentration, including validation studies and reagent blanks; the average and range of the test temperature(s).

(10) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(11) A description of any abnormal effects and the number of fish which were affected during each period between observations in each chamber, and the average concentration of test substance in each test chamber.

(12) Reference to the raw data location.

ES-8
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

FISH EARLY LIFE STAGE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TECHNICAL SUPPORT DOCUMENT FOR FISH EARLY LIFE STAGE TOXICITY TEST

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-11 which uses several species of fish to evaluate the effects of chemical substances on the early life stages of fish. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations are discussed. In addition, estimates of the cost of conducting the test are provided.
of-the-science developments.

II. Scientific Aspects

A. General

Toxicity tests with the early life stages of fish provide an extremely useful tool for the assessment of a chemical's potential to produce adverse environmental effects to aquatic organisms. An early life stage toxicity test is the logical sequel to an acute toxicity test, particularly where a high degree of toxicity has been demonstrated, or where continuous exposure is expected. Acute toxicity tests primarily measure mortality during a short duration (i.e. 96 hours) of exposure. The early life stage toxicity test is conducted to evaluate and estimate lethal and sublethal effects of low concentrations of a chemical or mixture over a longer period of exposure (i.e. 32 days or more), and during sensitive and critical stages

of fish growth and development.

The early life stage toxicity test can be used to assess the hazard potential to certain freshwater and estuarine fish resulting from chronic exposure to a chemical substance or mixture. The test procedures apply to nearly all types of chemicals that can be measured in freshwater and saltwater, and with slight modifications can be useful for testing other potentially toxic agents (e.g. oils, particulate matter, etc.).

It is generally recognized that fish are resources that are valuable ecologically, economically, recreationally and aesthetically. Healthy aquatic environments support a diversity of fish populations. A reduction in the quality of an aquatic environment will be indicated by effects upon the communities contained therein. Fish are considered for use in toxicity tests because they are sensitive indicators of chemical pollution, and are important, integral parts of aquatic communities and can be used as surrogates for other species in comparative toxicology.

The early life stage toxicity test can produce results which permit estimates of chronic toxicity normally derived from longer term, full life-cycle toxicity studies (Macek and Sleight 1977, McKim 1977). This conclusion was reflected by several investigators over the past decade who proposed the use of the early life stage toxicity test to predict chronic toxicity (Pickering and Thatcher 1970, Pickering and Gast 1972, McKim et al. 1975, Eaton et al. 1978, Spehar et al. 1977). McKim (1977) analyzed data from 56 life-cycle toxicity tests completed during the last decade with 34 organic and inorganic chemicals and four

species of fish, and concluded that the embryo-larval and early juvenile life stages were the most, or among the most, sensitive in their responses to chemical insult. Macek and Sleight (1977) concluded after a review of much of the available data that critical life stages (embryos and developing fry) exposed to toxicants provide estimates of chronically safe concentrations remarkably similar to those empirically derived from definitive chronic toxicity studies. In addition, it was stated that toxicity tests with critical life stages can provide information on potential long-term effects in situations where acute toxicity (i.e. 96-hr. LC50) is not observed.

Macek and Sleight (1977), stated that the concentration for most toxicants which will not be acutely toxic to the most sensitive life stages is generally the chronically safe concentration for fish. Carlson (1971) stated that several studies (i.e. Mount and Stephan 1967, 1969, Mount 1968, Brungs 1969, Eaton 1970, Pickering and Thatcher 1970, McKim and Benoit 1971) have shown significant differences between acute and long-term toxicity values. Allison and Hermanutz (1977) stated that freshwater fish populations could be directly damaged by prolonged exposure to a chemical at concentrations several thousand times lower than the concentration which causes acute mortality. The hazard of Kepone, for example, is greatly underestimated by acute toxicity tests (Hansen et al. 1977). It is obvious that assessment of the hazard potential of a chemical substance would be more accurate when using toxicity values derived from studies using sensitive, early life stages of fish and addressing chronic intoxication (Pickering 1974), rather

than the use of toxicity values derived solely from acute toxicity studies (i.e. 96-hour LC50 values). McKim (1977) also acknowledged the sensitivity of the early life stage toxicity test and proposed its use for screening large numbers of chemicals rapidly at a far lower cost per test than with a partial or complete life-cycle toxicity test.

B. Test Procedures

1. General

The basic concept behind the early life stage toxicity test is to estimate chronic toxicity through the use of a relatively short and inexpensive test. The literature reviews of McKim (1977) and Macek and Sleight (1977) are the most comprehensive evaluations of the early life stage toxicity test for use in evaluating chronic toxicity of chemicals to fishes.

The current experimental design incorporated into the early life stage toxicity test guidelines is derived from the various methods which have evolved historically, and reflect current state-of-the-science improvements. The first specific methods for use by government research and regulatory personnel were proposed in 1971 by the Committee on Aquatic Bioassays of the US Environmental Protection Agency and revised in 1972. The Bioassay Committee proposed a "Recommended Bioassay Procedure for Eggs and Fry Stages of Freshwater Fish." Another method by the Bioassay Committee was titled: "Recommended bioassay procedure for fathead minnow Pimephales promelas Rafinesque Chronic Tests," (USEPA 1972a). The Environmental Research Laboratories at Duluth (MN), Corvallis (OR) and Gulf Breeze (FlA) continue to play a major role in methodology development for aquatic toxicity

tests and have supplied valuable information in the development of the early life stage test guidelines. Investigative work reported in the literature at large has built considerably upon the early bioassay committee methods. In selecting the most practical and reliable test methods and procedures for the early life stage toxicity test guidelines, the available literature was reviewed to search for improvements or recommendations to the early committee methods. In addition, the American Society for Testing and Materials (ASTM) has been extremely active in developing a standard method for conducting early life stage toxicity tests. Many of the most prominent scientists in the field of aquatic toxicology are ASTM members with extensive practical experience in conducting early life stage toxicity tests. EPA has reviewed and considered the working drafts generated by the ASTM Task Group involved in the development of a standard practice for conducting toxicity tests with early life stages of fishes. The Agency has also solicited methods for conducting early life stage toxicity tests from several established testing laboratories for consideration in selection of the test standard methodology, as well as to ascertain practical contraints and deviations (in design and method) between commercial facilities (e.g. ABC protocols #7809 and #7810, EG & G Bionomics method 1979, and Union Carbide Environmental Services, March 1979).

2. Experimental Design

a. General

Many test design requirements were established upon the preponderance of available information supporting specific

methods or conditions. Occasionally the data available could not support selection of a specific requirement, and such requirements were selected following careful deliberation in order to minimize the number of possible variables which could affect the outcome or reproducibility of results. While lacking empirical support, such decisions provide for consistency in the test method which in turn enables comparison of test results with a higher degree of confidence. Selection of a testing requirement should always take into consideration the optimum conditions and requirements for the test species, based upon available information and research.

Early life stage exposure is defined by Macek and Sleight (1977) as exposure of a test species to a test substance during most, preferably all, of the embryogenic period (incubation of the fertilized eggs) and exposure of fry (as newly hatched fry through young juveniles) for a period of 30 days after hatching for warm water fish with embryogenic periods of 1-14 days (fathead and sheepshead minnows), and 60 days exposure after hatching for fish with longer embryogenic periods (i.e. brook and rainbow trout). The TSCA early life stage toxicity test guideline is initiated with newly-fertilized embryos (less than 48-hours old for the minnow species and less than 96-hours for the trout species). An attempt was made to ensure uniformity of exposure periods, and to permit the shipment of embryos to a testing facility which may lack in-house cultures of the test species. The 48-hour requirement ensures that warm water species (i.e. fathead and sheepshead minnows) will receive a minimum amount of exposure as embryos prior to hatching.

A minimum of five duplicated exposure concentrations of a test substance and at least one control should be used in conducting an early life stage toxicity test. This provides a minimum number of data responses to ensure statistical validity, as well as provide the minimum amount of responses to adequately develop the quantitative data necessary to establish biological conclusions and significance. The quantitative data derived from an early life stage toxicity test is used to determine the lowest toxicant concentrations which adversely effect the test fish and those which apparently do not. Five test concentrations with a minimum of one control have been used extensively and are statistically valid and historically acceptable. The preponderance of data developed in the field of aquatic toxicology has been generated using five concentrations with a minimum of one control.

The use of 5 test substance concentrations is designed to produce effects at the higher concentrations, while producing no observed effects in the lowest concentrations, with the mid-range concentration(s) contributing to the degree of certainty in the definition of the concentration-response curve (Doudoroff et al. 1951). It is obvious, however, that the desired responses of effect and no-effect are dependent upon how accurately the test substance concentrations are selected. Selection of the test substance concentrations for use in the early life stage toxicity test is considered on a case by case basis. A flow-through toxicity test at least 96 hours long using the larval or juvenile stages of the fish species to be tested should be performed. The highest concentration for the

early life stage test should be similar to the lowest concentration in the acute test causing a reduction in survival. The dilution factor recommended over the past decade has been primarily 0.5 between treatments. No hard and fast rule is possible for selecting the proper test concentrations. Caution should be exercised, because selection of the wrong concentrations and dilution factor can negate the acceptability of the test for its intended purpose by EPA. For example, if all treatment concentrations elicit a significant effect, the test probably should have been conducted at lower concentrations; conversely, if no significant effect occurs, higher concentrations probably should have been used.

The location of the test chambers and the introduction of test organisms into the test chambers are randomized to prevent biases from being introduced, and consequently affecting the objectivity necessary in the test. Biases can occur in environmental conditions (temperature, lighting, disturbances, etc.), embryo selection and distribution, diluter system function, etc.. Both the test organisms and test concentrations should be assigned to the exposure chambers by formal randomization, in order to avoid introduced biases.

b. Controls

Controls are required for every test to assure that any effects which are observed are due to the test substance and not to other factors. Similarly, the carrier (solvent for test substance) control demonstrates any stress induced by the carrier, apart from the test substance. Controls are universally required in toxicological studies and contribute

an essential value to the interpretation of test results, as effects observed in the test organisms exposed in test solutions can be attributed to the test substance only when evaluated in light of results from the controls.

c. Carriers

Carriers can affect (i.e. stress) test organisms and can possibly alter the form of the test substance in water. For these reasons the preferred standard carrier for a test substance is water, and the use of other carriers should be avoided unless absolutely necessary to get the test substance into water. A safety margin between the acute toxicity of the carriers and any possible subacute effects that might influence the experimental outcome is necessary. Triethylene glycol (TEG) and dimethyl formamide (DMF) appear to exert the least influence on test organisms and test substances of the several carriers that have been tested. Work performed by Rick Cardwell (unpublished data) indicates the following 96-hour, LC50 values (mg/l):

	Fathead minnow	Brook trout
TEG (Triethylene glycol) -	92,500	73,500
DMF (Dimethyl Formamide) -	10,410	8,366
Acetone	9,100	6,070

EG&G Bionomics (1979) state in their method for conducting an early life stage test with fathead minnows that the carrier concentration should not exceed one-one thousandth (1/1000) of the 96-hour LC50 for the species and

solvent. This would equate to 92 mg/l and 73 mg/l of TEG for the fathead minnow and brook trout respectively, based upon the above information.

Due to the low toxicity to aquatic animals, low volatility and a high ability to dissolve many organic compounds, dimethyl formamide and triethylene glycol are the preferred organic carriers for preparing stock solutions, but methanol, acetone, and ethanol may also be used. In order to determine the influence or possible effects caused by the carrier, a carrier control containing dilution water and the carrier at the highest concentration used in the toxicity test should be included and conducted simultaneously with the toxicity test.

Responses of control and carrier control fish provide a measure of the acceptability of the test, and it is for this reason and for use in interpreting the test results, that the controls are required. If a carrier is used, criteria for effects (e.g. embryo mortality, larva mortality, growth) used in the comparison of control and exposed animals should also be applied to the animals in the control and control with carrier chambers. If a significant difference exists in these criteria, the test may not be considered acceptable, as the data would inaccurately reflect the test substance toxicity, and thus the overall test results would be ambiguous. The overall survival of the control animals should be at least 80%; otherwise the test should be considered unreliable, because the effects which are observed cannot be attributed with confidence to the test substance alone.

3. Beginning the Test

It is important that the quality of the dilution water and the test substance concentration be constant at the time that the test is started, and for the duration of the test. A 48-hour observation period is required prior to beginning the test in order to ascertain that all equipment (e.g. temperature control, test substance delivery system, etc.) is functioning properly and to allow an equilibrium to be reached in the test system. An equilibrium is achieved when the test dilution water quality (e.g. dissolved oxygen, temperature, pH, etc.) is constant, and all physicochemical reactions, such as leaching of contaminants and adsorption of the test substance within the test system are stabilized. Fluctuation of the test substance concentration can be caused by adsorption of the test substance by the test system or by a malfunction in the test substance delivery system. The 48-hour observation period permits fine adjustments to the test substance delivery system and allows for any adsorption which may initially occur. Consistent test substance concentrations are critical for valid test results and should be accurately evaluated and maintained (Lloyd 1978).

Carmignani and Bennett (1976) demonstrated leaching of plastics from some closed aquaculture systems, and recommended that new aquaculture systems using plastics should be flushed for at least 10 days with fresh water at temperatures at least 3°C above the operating temperature of the system before they are put into service. Established systems should only require flushing for 48 hours to achieve an equilibrium.

After final calibrations of the test substance delivery system and verification of the actual test substance concentrations in the test water from each chamber, the test can be initiated with acclimated, normal appearing embryos. Since one of the measured parameters is hatching success, use of non-viable embryos would skew the test results and the data interpretation. In order to keep the embryos viable an exchange of water through the embryo cups should be maintained. This ensures a flow of oxygenated water over the incubating embryos and dislodges particles and debris which can encourage undesirable fungal growth. Static conditions contribute to fungal growth, thus maintaining water flow over incubating eggs has been standard practice since the earliest chronic studies with the fathead minnow. Mount (1968) used a rocker arm apparatus driven by a low speed (>1rpm <3rpm) electric motor, while other investigators (e.g. Hansen et al. 1977) have elected to use a self-starting siphon to vary the water level in the test chambers.

Each day until hatching the embryos are usually inspected without the use of a scope or magnifying viewer for the purpose of discarding obviously dead embryos. This is done in order to prevent fungus, which attacks dead embryos, from spreading to the surrounding live embryos. The use of a scope or viewer could unduly stress the developing embryos because of the amount of handling involved and is therefore discouraged. Handling of newly hatched larvae/fry is also discouraged in order to minimize stress and avoid injury.

Transfer of fry from embryo cups into the test chambers should not involve the use of nets; rather the fry should be allowed to swim out. This reduces the amount of stress and injury that could result from directly handling the fry. Trout incubated on screen trays will hatch out into test chambers and thus transfer is not necessary.

Daily records should be kept of the number of dead embryos and fry, and in addition, the number of unaccounted for or deformed embryos. Daily records are also made for any lethargic or glossly abnormal fry. This includes swimming behavior and physical appearance. The range of time-to-hatch (to the nearest day) for each replicate should also be recorded. These and other observations are detailed in Section 2.1.4: Observations and Measurements.

The number of embryos required to begin the test is flexible and varies upon the statistical power desired and the test species selected. Minimal standards of control response (e.g. percentage of embryos to hatch, percentage larval survival,and percentage overall survival) for each species dictates the number of embryos used.

Test guidelines for conducting early life stage toxicity tests specify only the minimum number of embryos required to enable the use of sensitive statistical analyses of the biological data.

4. Observations and Measurements

A final aspect of the test procedure pertains to the measurements and observations which should be made during the test. Daily observations are made to ascertain the functioning of the test equipment and to ensure the required test conditions are maintained (i.e. temperature, dissolved

oxygen, test substance concentrations, dilution water quality, etc.). Biological data responses are gathered through observations of mortality for embryos and fry, and for abnormal physical appearances and behavior. The biological responses are used in combination with physical and chemical data (i.e. test conditions) in evaluating the overall lethal and sublethal effects observed. The observations required during the test are the minimum intervals necessary to produce valid and interpretable data.

Generally, the most important data derived from observations in tests with the early life stages of fish are those that show significant differences between control treatments and experimental treatments based on the following: percent survival of embryos to hatching, time to hatching, survival of hatched fish to termination of test, time to swim-up for trout, survival from beginning to termination of test, and growth and development of young fish.

5. Acceptability Criteria

The acceptability criteria specified in the test guidelines are essential to the interpretation of the test results. The criteria are used to minimize and detect possible influences on the test results other than those produced by the test substance. The acceptability criteria also delineate the function of test conditions and parameters, and are based upon what has been normally achieved and observed in the preponderance of relevant studies reported in the literature.

Test acceptability is basically dependent upon the test results being primarily a function of the test substance,

and not due to the influence of external variables. For example, since various measured parameters of the test species (e.g. weight, length) are compared with the controls, it is important that controls are "normal" as determined historically. When control survival values are less than those typically observed in past tests, it can be assumed that there are problems with some aspect of the test (e.g. fluctuations in environmental parameters, stressed test organisms, etc.). Thus, test acceptability is dependent upon the criteria specified in the test guidelines which are designed to allow a determination of statistical validity, in addition to providing quantitative data necessary to establish biological conclusions and significance. Variation from the specified criteria indicates that other aspects of the test may be affected and final results can not be attributed entirely to the test substance. Thus, the usefulness of the test in evaluating the hazard potential of a test substance is of questionable value unless the acceptability criteria are satisfied.

6. Test Results and Analysis

a. General

In conducting toxicity tests with the early life stages of fish, data are obtained to determine the effects of the test substance on a particular test organism during a sensitive period of its growth and development. As in all aquatic toxicity tests, statistical hypotheses are developed and the experiments are designed to test these hypotheses. Experimental data are gathered, analyzed and statistical conclusions are drawn. It should be remembered, however, that statistical hypotheses and subsequent conclusions are

measurements of broader biological hypotheses and biological responses and interpretations. Hence, a statistically significant conclusion based upon a preselected significance level does not necessarily signify a similar or equivalent biological significance. The biological significance and interpretation of a statistically derived conclusion should be based upon the experience and professional judgment of the investigator or reviewer.

Toxicity tests with fish early life stages are generally used to determine the lowest test substance concentration(s) which adversely affect the test species, and those which apparently do not.

These are usually defined as:

Lowest Observed Effect Concentration - The lowest test concentration from a valid toxicity test for which the null hypothesis of no difference (i.e. control and treatment means were similar) was rejected ($P<.05$) for any specified response.

No Observed Effect Concentration - The highest test concentration from a valid toxicity test for which insufficient evidence exists to reject the null hypothesis of no difference ($P< 0.05$) for any specified response.

The proposed early life stage toxicity test guidelines are specific only where minimum parameters and conditions are recognized to be essential for the production of valid results. The flexibility of the test guidelines allows for the wide variations among commercial testing facililties. For example, the number of tanks and fry chambers or embryo cups is not mandatory. While the majority of facilities

utilize the minimum number of test substance concentrations (i.e. 5 plus controls), there is little uniformity in the number of embryos used to initiate a test (e.g. the typical range is from 850 to 2800). Each test can produce valid and acceptable results, yet specific guidance for the analysis of data derived is difficult. Each different combination of fry chambers, embryo cups, etc., would require a different design, and therefore would require a separate analysis.

b. Analysis

Various methods for the analysis of data are available and acceptable. The following discussion is an example of an acceptable approach. The key to selecting an appropriate experimental design and accompanying statistical analyses lies in the identification of the smallest division of experimental material such that any two units may receive different treatments in the actual experiment (Cox 1958). Data from the toxicity tests are usually either continuous (e.g. length or weight measurements) or dichotomous (e.g. number hatching or surviving) in nature. In general, continuous data should be analyzed using an appropriate analysis of variance (ANOVA) technique followed by an appropriate multiple comparison test. Dichotomous data should be analyzed using a form of 2x2 contingency table.

Several reference sources are available to determine which ANOVA is the most appropriate (Sokal and Rohlf 1969, Box et al. 1978). If solvent and dilution water controls are used in the toxicity test, they should be evaluated for possible combining. This should be done using a "T-test" with a large probability level (e.g. $P < 0.25$). If the null hypothesis is rejected, the responses in both controls

should be evaluated and the most appropriate control used in all further data analyses. As a part of the ANOVA it is desirable to plot the residual (error) versus concentration, to determine whether there have been any obvious violations of the assumption of normality. The following should be presented when reporting the results from an ANOVA technique: (a) the ANOVA model; (b) the ANOVA table; (c) the F statistic; and (d) the level of significance for the F statistic. It is also appropriate to report any residual plots generated. The ANOVA is only the first step; assuming the ANOVA F test was sigificant ($P< 0.05$), a comparison is made of all non-zero concentration responses with the control response. The appropriate procedure for this is normally a multiple comparison test such as Dunnett's procedure (Dunnett 1955, 1964). Dunnett's procedure is commonly used and is analagous to performing +/ - tests on the control with each concentration tested. A second technique is William's procedure (Williams 1971, 1972). This procedure also compares the control with each test concentration, but with the assumption that the true mean response decreases (or increases for certain types of response) with increasing concentrations. If this assumption is correct, then William's procedure is the more powerful statistical technique. An excellent discussion of these techniques and other multiple comparison procedures is provided by Chew (1977).

Presentation of results from these tests should include the critical value of the statistic and its corresponding level for each comparison of the test statistics. Dichotomous data should be analyzed using a 2x2 contingency

table. When attempting to distinguish between a control response and the same response (e.g. hatchability) at a low test substance concentration level, the calculated expected cell frequencies are likely to be low. Therefore, it is appropriate to analyze these data using Fisher's exact test (Sokal and Rohlf 1969, Fisher 1958) rather than with an approximation test like the Chi-square test. If two control treatments (e.g. regular and carrier) are used in the toxicity test, they should be evalulated using a 2x2 contingency table rather than the T-test.

C. Test Conditions

1. Test Species

a. Selection

Four species of fish are proposed for use in conducting early life stage toxicity tests. These species are:

1) Fathead Minnow (Pimephales promelas Rafinesque)
2) Sheepshead Minnow (Cyprinodon variegatus)
3) Brook Trout (Salvelinus fontinalis Mitchill)
4) Rainbow Trout (Salmo gairdneri)

These species are representative of warm and cold freshwater and estuarine fish respectively.

There are several criteria to be considered when selecting a fish species for use in an early life stage toxicity test:

1) Embryos are required to initiate the early life stage toxicity test; therefore recently fertilized eggs or fresh sperm and ova should be readily available. As such, it is desirable that the species be amenable to laboratory conditions, demonstrating high survival in captivity.

2) The species should possess a demonstrated ability to reproduce readily in close confinement, producing large numbers of eggs, or they should readily available from reliable commercial hatcheries.

3) The species should mature rapidly, yet be small enough in size to enable the use of large (statistically valid) numbers during the toxicity tests.

4) The species should be relatively widely distributed, with non-polluted areas included in its geographical range.

5) The species should be relatively sensitive to toxic pollutants, and its susceptability to toxic pollutants should equal or exceed that of other fish species in its geographical range.

The four species selected for use in conducting early life stage toxicity tests generally satisfy these criteria, and can be used individually to address practical problems which may be restricted to a specific geographical location. Each species has been used successfully in early life stage toxicity tests, and in fact, have been used more often than any other fish species for such test purposes. In addition, these species have also demonstrated success in both acute and full chronic toxicity tests. The use of the same species for many aquatic toxicity tests provides for a good comparative toxicological data base. Additional support for the selection of these particular species is provided in the following discussions.

Fathead Minnow (Pimephales promelas Rafinesque)

The fathead minnow is an important forage fish (Scott and Crossman 1973), which has significant value as a

converter of algae, organic detritus from bottom deposits and planktonic organisms. It is widely distributed, small, highly prolific and has a prolonged spawning period, assuring availability of recently hatched and juvenile fatheads to a large variety of predators (Scott and Crossman 1973). A list of predators for the fathead minnow would require a listing of all of the carnivorous fish and fish-eating birds associated with it throughout its geographic range (Scott and Crossman 1973). Its role as an important forage species makes it an essential link in the food economy of some natural ecosystems and its value in this role is inestimable (Scott and Crossman 1973).

The fathead minnow is distributed widely throughout the Great Plains region of Canada and the United States, as well as much of the region east of the Great Plains from the southern drainage of the Hudson Bay and the Maritime Provinces of Canada southward through the Ohio and Cumberland systems to the Tennessee River Basin. Although apparently absent on the Atlantic slope and the Gulf states east of the Mississippi River, the fathead is present as far west as New Mexico and Chihuahua, Mexico in the South. It has also become established in California (Blair et al. 1968, Scott and Crossman 1973).

The fathead minnow has been established by many laboratories as an assay fish for the determination of the toxicity of aquatic pollutants (Manner and DeWese 1974). It is considered a good bioassay animal (Mount 1973) and has been used successfully in many bioassays (Martin 1973, McKim 1977). It has a demonstrated ease of spawning and handling, reaching sexual maturity in five months under the proper

conditions (Adelman and Smith 1976). The time to hatching depends on temperature, but the average is 4.5 - 6 days; at 25°C, 5 days is normal (Scott and Crossman 1973). Newly hatched fry are about 5 mm long, and reach an average length of 50-70 mm as adults (Scott and Crossman 1973). The early embryology of the fathead minnow has been studied by Manner and DeWese (1974), who also indicated the extensive use of the fathead in toxicity studies. McKim (1977) considered the fathead minnow an example of a consistently sensitive species for use in toxicity studies. These attributes and characteristics in combination with the proven ability to easily transport embryos and fry, make the fathead minnow an appropriate selection for use in early life stage toxicity tests.

Sheepshead Minnow (Cyprinodon variegatus)

The sheepshead minnow is an omnivorous killifish (Family Cyprinodontidae) which occurs naturally in estuaries from Cape Cod, Massachusetts to Yucatan, Mexico and in the West Indies to northern South America (Hildebrand 1917, Blair et al. 1968). The sheepshead minnow inhabits tidal ponds, sloughs, saltwater creeks, bayous and bay shores (Simpson and Gunter 1956) and while prefering estuarine environments has also been observed in freshwater streams and in strictly saltwater (Hildebrand 1917). The sheepshead minnow is considered an ecological dominant in its environment, and is very hardy, pugnacious, and tolerant of salinity fluctuation (Simpson and Gunter 1956). It is a very prolific species and its fecundity helps to explain its great abundance (Hildebrand 1917, Simpson and Gunter 1956). It has value as a converter of detritus and vegetable matter and in

controlling mosquito larvae, but its greatest value probably lies in the food source it provides for larger fishes and other predators. These include: spotted seatrout (Cynoscion nebulosus), yellow bass (Micropogon undulatus), red drum (Sciaenops ocellata), black drum (Pogonias cromis) and many others (Gunter 1945, Darnell 1958).

Hildebrand (1917) stated the spawning period for the oviparous sheepshead minnow was considerable, ranging from March until October. The incubation period at laboratory temperatures ranges from 5 to 6 days (Hildebrand 1917), with 5 days at 30°C being normal (Schimmel and Hansen 1974). The sheepshead minnow spawns readily in laboratory aquaria, is fecund and has a short life cycle and survives well under laboratory conditions (Schimmel and Hansen 1974, Hansen and Parrish 1977). The combination of its size and hardiness make it a useful fish for laboratory toxicity tests (Schimmel et al. 1974, Schimmel and Hansen 1974, Hansen and Parrish 1977, Parrish et al. 1977). It should be noted, however, that several of these authors have indicated that the sheepshead minnow is relatively insensitive and that its reproductive strategy differs from most marine species, both are drawbacks which make the sheepshead minnow less than ideal. However, no other marine or estuarine species has a standardized method available for conducting early life stage tests.

Brook Trout (Salvelinus fontinalis Mitchill)

The brook trout is considered an important resident of many lakes, streams and ponds across the US, as both a native species and introduced species (Thatcher et al. 1976, Larson et al. 1977). Under natural conditions this North

American species occurs along the northeastern North American Seaboard south to Cape Cod, in the Appalachian Mountains southward to Georgia, west in the upper Mississippi and Great Lakes drainages to Minnesota, north to Hudson Bay (Scott and Crossman 1973). In Canada the brook trout is widely distributed throughout the Maritime Provinces, and it has been introduced widely and often successfully into many parts of the world because of its appeal as a sport fish (Scott and Crossman 1973). Included in its introduced range are the higher mountainous parts of Western North America. These authors indicated that brook trout occur in clear, cool, well oxygenated streams and lakes and that a long interest in brook trout as a hatchery-reared and pond-cultured sport fish has resulted in an accumulation of information on the species. Thatcher et al. (1976) stated that its life cycle and laboratory culture is well documented.

Brook trout are carnivorous and feed upon a very wide range of organisms, including worms, leeches, crustaceans, aquatic insects, terrestrial insects, and a number of fish species (Scott and Crossman 1973). These authors indicate that the most serious predators are fish-eating birds (i.e. kingfishers and mergansers) and larger fish such as the rock bass.

The brook trout eggs are large, 3.5-5.0 mm in diameter, and incubation time is dependent upon such factors as temperature and oxygen; at 41°F (5°C) eggs hatch in about 100 days, at 43°F (6.1°C) in about 75 days, and at 50°F (10°C) in 50 days (Scott and Crossman 1973). The long incubation period of this species necessitates that the

duration of the early life stage life stage toxicity test be correspondingly longer in comparison to the early life stage toxicity tests conducted with the warm water minnow species.

Rainbow Trout (Salmo gairdneri Richardson)

The rainbow trout is the most common salmonid used for bioassay purposes, is the easiest to rear under laboratory and commercial hatchery conditions, and is easy to spawn artificially to obtain eggs for testing. Brood stock can be retained for several years with good success. The eggs are easily transported from commercial hatcheries, with good hatching success and survival of young. The rainbow is native to the West Coast and Rocky Mountains of North America and is now widespread over most of the United States. Several early life stage toxicity tests have been completed with the rainbow trout with very good success. The relatively rapid embryo and larval growth, along with its relative resistance to disease compared to other salmonids, makes it an ideal cold water fish for testing toxic materials.

b. Sources

It is preferable to maintain in-house brood stock for obtaining sufficient quantities of test embryos of known history, quality and age; however, eggs and sperm or newly fertilized embryos can be obtained from a reliable external commercial or research facility. This is necessary to prevent restricting the number of testing facilities to only those which possess in-house cultures.

Fathead minnow embryos are generally obtained through natural spawning under laboratory conditions. An artifical spawning substrate for use in culturing fathead minnows is

described by Benoit and Carlson (1977). The Duluth Environmental Research Laboratory has maintained fathead minnow culture stock successfully for many years.

Sheepshead minnow embryos can be obtained through natural spawning or artifically through hormone injections and stripping eggs and excising male testes. These methods are discussed in Bioassay Procedures for the Ocean Disposal Permit Program (EPA 600/9-78-010) and by the following investigators: Schimmel and Hansen (1974) and Hansen and Parrish (1977).

Rainbow and brook trout embryos can be obtained through natural spawning (Hokanson et al. 1973, USEPA 1972b) or by dry artificial methods. Dry artificial techniques are valuable because brood stock can be artificially stripped and returned to their ponds or chambers without harm. The eggs can be immediately fertilized, left for an hour or two to water harden, and then be transported to the laboratory. Eggs obtained from dry artificial techniques can be gathered in quantity, fertilized at the same time and shipped chilled or iced down with no adverse effect on viability. The eggs become increasingly sensitive approximately four days post-fertilization, and should not be moved between day 5 and day 14. The eggs can be moved carefully after the eyes become visible, as the sensitive embryo period occurs prior to day 14. Therefore, testing should start with eggs which have been fertilized (embryos) and are less than 4 days old. Natural spawning is less desirable as it is difficult to obtain a sufficient quantity of embryos necessary to conduct the toxicity test which are uniformily less than 96-hours old.

2. Maintenance of Test Species

a. Acclimation

The health and quality of the test organism is all-important in any toxicological investigation (Hunn et al. 1968, Brauhn and Schoettger 1975), and acclimation is of direct importance for correctness of bioassay results (Sprague 1969). Acclimation of embryos to the test dilution water and temperature is necessary to minimize or avoid stress at the initiation of the toxicity test. Results of tests with stressed organisms will not accurately represent the response of the test species to the test substance alone.

In-house cultures for the production of a test species (i.e. fathead minnow) should be maintained at or very near the actual test temperature and should utilize test dilution water. In such cases acclimation is easily accomplished, and stress producing factors are minimized.

Embryos received from external sources should be acclimated to the test temperature and dilution water. This should be accomplished quickly for transported fathead and sheepshead minnows, because generally they will be received by the testing facility at close to 48 hours of age and will commence hatching within a day or two. Acclimation of rainbow and brook trout embryos may require a longer period of time, as they are generally transported with ice and rapid temperature fluctuations should be avoided (Brungs and Jones 1977). However, the longer incubation period of both trout species makes the acclimation period prior to the initiation of the test less critical.

b. Feeding

The time to first feeding and the amount and type of food is dependent upon the test species. Feeding of fathead and sheepshead minnows should commense when newly hatched larvae are transferred from embryo cups into the test chambers. This is because some of the larvae will be 48 hours old at that time, and feeding is necessary to provide nourishment for the young fish which were sustained previously by their egg-sacs. Schimmel and Hansen (1974) noted that sheepshead minnows accept live brine shrimp nauplii 48 hours after hatching. The practice of feeding fathead minnow fry at 48-hours was included in the early EPA methods for partial and full chronic bioassays (USEPA 1972a,b), and is practiced by the majority of investigators using both minnow species for early life stage toxicity tests. Investigators have also supplemented a wide variety of additional foods such as daphnia, trout pellets, dry flake and frozen commercial preparations after the first week. Mehrle et al. (1977) demonstrated the effect and importance of diet quality on the outcome of toxicological research. In order to minimize the effect of various diets on the outcome of the toxicity test, the recommended food source is live brine shrimp nauplii Artemia salina. H.T. Smith et al. (1978) studied the effects of population density and feeding rate on the final population density, growth and fecundity of fathead minnows. It was found that length and weight of fish increased with increased food availability. As such, the fathead and sheepshead minnow larvae should be fed at least 3 times a day during the week and twice on the weekend for the duration of the study.

This recommendation is felt to adequately satisfy the nutritional requirements of the minnow species, but does not constitute an unnecessary requirement nor expense in the test. An automatic brine shrimp feeder is discussed by Schimmel and Hansen (1975), which could be employed on weekends.

The brine shrimp should be analyzed for contaminants, and contaminant levels should not exceed the specified criteria for dilution water for maximum contaminant concentrations, in order to minimize the possible effects on the test species. Contamination of food by pesticides, PCB's, phthalates, mercury, lead or other ubiquitous substances would render the test results ambiguous.

Trout feeding requirements are different, in that the yolk sac of newly hatched trout generally takes between 32 and 38 days to be absorbed depending upon temperature (Atchison and Johnson 1975, Mauck et al. 1978). Therefore feeding should commense at swim-up. Trout starter food should be fed ad libitum five times a day, with the tanks cleaned daily. This is in accordance with the early EPA (1972b) partial chronic test method, with indicates trout alevins and early juveniles should be fed trout starter food a minimum of 5 times daily.

Fish are not fed for the 24-hour period prior to termination of the test in order to minimize the effect consumed food may have on analysis of fish weight.

3. Facilities

a. General

The facilities needed to perform an early life stage toxicity test as prescribed in the TSCA test guidelines

include: (1) flow through test chambers (tanks) for exposing embryos and fry; (2) embryo cups or screen trays for holding the embryos until hatching; (3) test substance delivery system; (4) a mixing chamber to promote mixing of the test substance and dilution water; (5) a mechanism for controlling and maintaining water at specified temperatures; (6) apparatus capable of controlling the photoperiod at specified regimes; (7) a device for removing particulate matter and gas bubbles and for aerating the dilution water as necessary; (8) suitable apparatus (e.g. magnifying viewer) for examining embryos; (9) apparatus for precise measurement of fish lengths and weight; and (10) facilities for providing the necessary food (i.e. live brine shrimp nauplii); and (11) facilities (or access to facilities) for performing the required water quality analyses.

b. Construction Material

All pipes, embryo cups, screen trays, mixing chambers, metering devices, and test chambers should be made of materials that minimize the release of chemical contaminants into the dilution water or the adsorption of test substances. Chemicals that leach from construction materials may be toxic to test organisms or they may act synergistically or antagonistically with test substances thereby producing inaccurate results. Leaching of undesirable substances from perfluorocarbon plastic, #316 stainless steel, and glass, and adsorption of test substances to these materials is minimal. Rubber, copper, brass, galvanized metal, lead and epoxy resins should not come in contact with dilution water, stock solution, or test solutions because toxic substances they contain may leach into those media.

All containers and pipes coming in contact with the dilution water need to be conditioned before use in order to leach out and wash away undesirable residues that may be present.

When cast iron is used in freshwater systems, rust may develop and its removal by strainers is recommended to prevent fouling of the diluters and delivery systems.

c. Test Chambers

Test chambers, delivery systems, pipes or tanks exposed to solutions that may come in contact with test organisms should not be soldered or brazed. The materials used in soldering or brazing contain lead, tin, copper, or zinc which may leach into the solution and may be toxic to the test organisms (Pickering and Vigor, 1965). Instead, metal parts should be welded or bonded with clear silicone adhesive, the preferred bonding agent for all construction materials. This adhesive is inert, and the acetic acid which it releases is easily washed away or volatilized from the system. However, the amount of adhesive which contacts any test solution should be minimized because it may adsorb test chemicals. It should be applied to the outsides of chambers and apparatus to minimize contact with the dilution water and test substance. Epoxy glues are not recommended for use because they may contain unreacted toxic monomers; if it is necessary to use epoxy glues, allow a considerable curing time prior to use.

Many different sizes of test chambers have been used in early life stage toxicity tests. There is no present justification for requiring specific dimensions, and the size of the test chambers may be considered acceptable if

the specified flow rate and loading requirements can be achieved.

d. Embryo Cups and Screen Trays

Embryos used to initiate early life stage toxicity tests are exposed to the test substance during incubation in embryo cups or on screen trays, where they can be carefully observed and protected, and the optimal conditions to ensure hatching success can be provided. Embryo cups are usually glass retaining vessels (or similar sized sections of polyethylene tubing) with stainless steel or nylon screen bottoms or petri dishes with nylon screen or stainless steel sides (Mount and Stephan 1967; Mount 1968).

Various modifications of embryo cups have been used successfully to incubate the embryos during toxicity studies. Acceptable embryo incubator chambers for trout have been described by McKim and Benoit (1971). Trays of stainless steel screen with the sides turned up and supported above the bottom of the exposure chamber (e.g. by folded screen legs) are also acceptable, and have been used successfully at the Environmental Research Laboratory at Corvallis, Oregon.

Embryo cups should be labeled to facilitate identification and tracking of embryos with the various test substance concentrations. The cups should be suspended or arranged in a test chamber to ensure that the test solution flows regularly into and out of each cup and that the embryos remain submerged. Test substance delivery systems or test chambers or both should be constructed so that the embryos are not stressed by turbulence. As indicated

previously, embryo cups can be oscillated by a rocker arm apparatus (Mount 1968), or the water level in the test chambers varied by means of a self-starting siphon (Hansen et al. 1977). This oscillation serves to ensure a regular flow of water through the embryo cup, which provides oxygen needed by the developing embryos. It also helps to remove debris, which in combination with stagnant water conditions contributes to fungal infestation.

e. Test Substance Delivery System

In order to maximize the accuracy and precision of data developed for use in evaluating the hazard a chemical presents to the environment, it is necessary to minimize variability in the testing procedure to the extent that such reductions in variability are cost effective. To accomplish this, the quantity of the test substance introduced by the test substance delivery system should be constant as as possible from one addition of test substance to the next. Fluctuation in the quantity of test substance introduced into the test chamber may affect the validity of the test results more significantly than fluctuations in other test conditions. The greater the variation in the quantity of the test substance introduced, the greater the abnormalities and spread of the response values; hence, the need to calibrate and verify the calibration of the test substance delivery system. Calibration includes determining the flow rate through each chamber, and the proportion of stock solution to dilution water delivered to each chamber. Variations in the quantity of dilution water entering the test chamber during a given time interval also may create undesirable differences in test conditions from one test

chamber to another. The concentrations of dissolved oxygen and test substance in a test chamber, for example, may decrease more rapidly in chambers having lower flow rates. Metabolic products of the test fish, such as ammonia, and products resulting from the degradation of the test substance, may also accumulate to a greater extent in chambers with lower flow rates. Differences from test chamber to test chamber in the concentration of dissolved oxygen, test substance, metabolic products and degradation products, individually or in combination, may also result in response values for the test organisms which are inaccurate or which lack an adequate level of precision. Flow rates through test chambers should not vary by more than 10% from any one test chamber to any other. The delivery system operation should be checked daily for normal operation throughout the test. This is extremely important for accurate interpretation of data results (Lloyd 1978)

Many test substance delivery systems are referenced in the literature at large (Lemke et al. 1978) and can be selected depending upon the specific characteristics and requirements of the test substance. Proportional diluters (Mount and Brungs 1967) are suitable for extensive use, and have the following advantages: (1) timing problems are minimal; (2) operation is simple and easy to understand; (3) malfunctions are infrequent; and (4) a series of concentrations can be delivered, each as much as 90% of each preceeding concentration. The main disadvantage is that it is impractical to deliver a series of concentrations with a dilution factor greater than 50% between concentrations (Mount and Brungs 1967). In addition, proportional diluters

are not commercially available everywhere and may require ceilings more than eight feet high.

Many modifications to delivery systems have been recommended by various researches, including: Brungs and Mount (1970), McAllister et al. (1972), Schimmel et al. (1974), and DeFoe (1975). Other relevant systems are discussed by Freeman (1971), Chandler et al. (1974), Chandler and Partridge (1975), Smith et al. (1977) and Abram (1973). Most delivery systems are operated by the hydrostatic force of dilution water or the test solution in a system of siphons (with and without float operated values), or by electro-machanical means. Metering pump systems (Garton 1980) can be simple, compact, easy to set up, portable, and relatively accurate if high quality pumps are used. Such pumps will last a long time with proper maintenance.

Proportional diluters and metering pumps have the capability of delivering consistent quantities of a test substance and dilution water to the test chambers, and have been used extensively in aquatic toxicity tests. However, different delivery systems which incorporate proportional diluters or metering pumps exhibit individual idiosyncrasies. Due to possible malfunctions caused by such idiosyncrasies, it is necessary to calibrate each system to verify the concentration of the test substance and the volume of dilution water which each system actually delivers.

A small chamber to promote mixing of toxicant-bearing water and dilution water should be used between the test substance delivery system and the test chambers for each

concentration. Benoit and Puglisi (1973) describe such a flow-splitting chamber to promote thorough mixing. When a single proportional diluter is used, separate delivery tubes from the mixing chamber to each duplicate test chamber are necessary to delivery accurate and identical concentrations of the test substance.

Particulate material and gas bubbles should be removed from the test system. Test substance concentrations may be altered by sorption to particulate matter or by volatilization due to excessive gas bubbles. Both particulate material and gas bubbles may clog the diluter system. To avoid this problem, an apparatus capable of removing particulate matter or gas bubbles or both from the dilution water is recommended (USEPA 1975, USEPA 1972a).

f. Cleaning

Before use, test systems are cleaned to remove dust, dirt and other debris and residue that may remain from previous uses of the system. Any of these substances may affect the results of a test by sorption of test materials or by exerting an adverse effect on the test organisms. New chambers are cleaned to remove any chemical or dirt residues remaining from manufacture or accumulated during construction and storage. Detergent is used to remove hydrophobic or lipid like substances. Acetone may be used for the same purpose, and as a final rinse. It is important to use pesticide-free acetone to prevent the contamination of the chambers with pesticides which may be toxic to the test organisms or which might otherwise influence the outcome of the test. Hypochlorite at 200 mg/liter is useful for removing organic matter and for disinfection. A

solution containing 200 mg hypochlorite/liter is made by adding 5 ml. of household bleach (chlorine) to 1 liter of water. However, acid and hypochlorite should not be used together because hazardous fumes may be produced. Acid (e.g. 5% nitric acid) is useful for removing mineral deposits, metal residues and bases from the system.

For example, a new system would be typically cleaned by washing with detergent and rinsing with water, reagent-grade acetone, water, acid (e.g. 5% nitric acid), and twice with dilution water.

At the end of a test, if the test substance delivery system, embryo cups or test chambers are to used again, they should be: (a) promply emptied; (b) rinsed with water; and (c) cleaned by a procedure appropriate for removing the substance tested as well as other debris. It is easiest to clean the equipment immediately following the test termination, before chemical residues and organic matter become embedded or absorbed. Conditioning should be considered an important part of cleaning.

g. Dilution Water

Variations in water quality parameters have been found to influence the results of toxicity tests involving aquatic organisms (Tucker and Leitzke 1979). For instance, Mount (1968) and Mount and Stephan (1969) conducted experiments to determine the acute and chronic toxicity of copper to the fathead minnow in hard and soft water. The dilution water used in each test was controlled to maintain nearly constant hardness, alkalinity and pH. In addition, temperature was maintained within a narrow, optimum range. Results of these tests indicated that hardness, and to a lesser extent, other

characteristics such as pH, acidity, and alkalinity, affected both acute and chronic toxicity of copper. Brungs et al. (1976) stated that many environmental factors influence the toxicity of copper and cited several authors that documented effects of oxygen, temperature, hardness, turbidity, carbon dioxide, magnesium salts, organic compounds, nitrilotriacetic acid and spent sulfite liquor. Carroll et al. (1979) stated that many workers have reported that heavy metals are less acutely toxic to aquatic organisms in hard water than in soft. While data on all the possible effects of various water chemistry parameters on the toxicity of chemicals to fish are very incomplete, those which are available show clearly that variations in certain water chemistry parameters may cause variation in the results of toxicity tests with fish. In view of the Agency's need for accurate data in evaluating the hazard of a chemical and the risk it poses to the environment, the dilution water used in performing toxicity tests should show evidence of consistency in its chemical make-up.

A dependable source of clear surface or ground water will usually have a greater consistency in its chemical make-up than a municipal water supply. Municipal water may originate from several sources which may differ considerably. In addition, municipal water frequently is treated chemically as part of a purification process. Since the proportions in which water from different sources are mixed, and since the chemical treatment given water during the purification process may be different from time to time, the chemical make-up of municipal water may vary considerably.

The long duration of the test, and the large volume of water required for flow-through conditions economically prohibit the use of reconstituted water (freshwater or saltwater).

Fish culturists do not know all of the conditions required to maintain fish health, nor do they know all of the components in water that adversely affect the health of fish (Brauhn and Schoettger 1975). Nevertheless, to avoid possible inconsistencies and inaccuracies in test results, minimal variability and healthy fish are needed for toxicity tests. There is, therefore, a need to determine that the dilution water is capable of suporting the fish species to be tested in a healthy condition for the duration of the test period.

An appropriate way to make such a determination is to place young fish of a sensitive species, preferably the one to be used in subsequent tests, in the dilution water for an extended period of time and observe their behavior, growth and development. Ideally, such observations would be made by experienced fisheries biologists familiar with certain stress reactions which are difficult for an untrained observer to identify (Brauhn and Schoettger, 1975). As an indication of the uniformity of the dilution water, it is recommended that certain water chemistry parameters be measured at least twice a year, or more frequently (as specified) if it is suspected that one or more of those parameters has changed significantly. The water chemistry parameters and the maximum acceptable concentrations for contaminants specified in the test guidelines are those considered not to adversely affect fish (USEPA 1973, APHP,

AWWA, WPCF 1975). Recognizing that some variation in water chemistry is normal in natural freshwater, a 10 percent fluctuation from month to month in water hardness, alkalinity and conductivity and a variance of 0.4 pH unit is considered acceptable. Variations in excess of the concentrations or values cited in the standard may alter the value of the data developed in the toxicity test, and render the results ambiguous.

The quality of dilution water is also important for toxicity tests with salt water organisms (Bahner et al. 1975). Dilution water for conducting early life stage toxicity tests with the sheepshead minnow is considered to be of constant quality if the minimum salinity is greater than 15°/00 and the weekly range of salinity is less than 15°/00. The monthly pH range should be less than 0.8 unit (USEPA 1978, Hansen and Parrish 1977, Hansen and Schimmel 1975, Schimmel et al. 1974).

Specially designed systems are usually necessary to provide seawater from natural sources, and the water should be filtered through a pore size less than 20 micrometers in order to remove larval predators (Parrish et al. 1977, Goodman et al. 1976, Bahner et al. 1975, Schimmel et al. 1974, USEPA 1978).

The dilution water (fresh or salt) should be intensively aerated by such means as air stones, surface aerators, or screen tubes (Penrose and Squires 1976, Rucker and Hodgeboom 1953) before introduction of the toxicant. Aeration should not be employed after the test substance has been introduced since the process of aeration may result in a loss of the test substance through volatilization. Adequate aeration of

the dilution water will help bring the pH and the concentration of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and effect removal of volatile compounds which may be present.

A variety of microorganisms are found in many water supplies. Their presence in dilution water may be undesirable as they may be infectious to the test fish. Dilution water that may be contaminated with such undesirable microorganisms should be passed through membrane filters or an ultraviolet sterilizer equipped with an intensity meter (Bullock and Stuckey 1977). Efficacy of the sterilizer may be determined by using standard plate count methods (APHA, AWWA, WPCF 1975).

Additional parameters cannot be specified for freshwater dilution water quality, because of the wide geographical distribution of available testing facilities. Specific requirements for hardness, etc., would preclude those facilities which failed to meet such requirements.

4. Environmental Conditions

a. Dissolved Oxygen

Fish embryos and developing larvae are particularly sensitive to deficiencies of oxygen (Doundoroff and Shumway 1970). Exposure of fish to a test substance may increase the rate at which they consume oxygen, with a resulting rapid uptake of dissolved oxygen. Some test substances undergo oxidation when introduced into the test chamber. This may result in a chemical or biochemical oxygen demand, depending on the nature of the oxidation which removes dissolved oxygen from the surrounding water.

Microorganisms present in the test chamber also may create a demand for the dissolved oxygen. The normal oxygen needs of the fish, with possible chemical or biochemical oxygen demands of the test substance,and oxygen consumption by micoorganisms, individually or in combination, could reduce the level of dissolved oxygen below that required by the test fish. Oxygen to replace that which has been depleted can be provided by increasing dilution water flow rate.

The concentration of dissolved oxygen in the dilution water should be between 90% and 100% saturation, the optimal range for all the test species. A minimum level of 60% saturation of dissolved oxygen has been recommended by earlier groups and committees for test solution water used in early life stage toxicity studies (APHA, AWWA, WPCF 1975, USEPA 1972a,b). This minimum level of dissolved oxygen is required in order to avoid possible variations in test results due to the effects of anoxia on the test fish. Doudoroff and Shumway (1970) noted that there is very little agreement in the literature on the reported findings of the effects and minimum requirements of dissolved oxygen for fish. These authors noted that pertinent information on the effects of low concentrations of dissolved oxygen on fecundity and embryonic development is also very limited; but embryos of the fathead minnow appear to be adequately protected from stress at oxygen concentrations above 5 mg/l at temperatures normal for this species. Similarly, Brungs (1971b) studied the chronic exposure of fathead minnows to continuous reduced levels of dissolved oxygen in order to evaluate effects on reproduction, fry growth and fry

survival. Results indicated a statistically significant reduction in fry growth at all concentrations below the control (7.26 mg/l), and that time to hatch was increased at each successively lower dissolved oxygen concentration. No effects were observed on percentage hatch. The highest dissolved oxygen concentration in this study (other than the control) was 5.0 mg/l; this indicates that 60% dissolved oxygen saturation level appears to be somewhat less than an ideal minimum concentration for early life stage toxicity tests. These data support the requirement of a minimum level of dissolved oxygen for the test species specified in the early life toxicity test guidelines, especially since the minimum dissolved oxygen requirements of warm water species are generally not as critical as those for cold water species (Doudoroff and Shumway 1970).

b. Flow Rate

The flow of the water through the test chambers minimizes the accumulation of metabolic products such as ammonia which, if allowed to accumulate, could reach concentrations lethal to the fish or alter their sensitivity to test substances. The build-up of organic matter within the exposure chambers might provide a nutrient source for bacteria present in the water. Bacteria using oxygen to metabolize and decompose the organic matter in the tanks could reduce the dissolved oxygen concentrations of the water. Decreased dissolved oxygen concentrations as well as the accumulation of toxic metabolic products could increase the likelihood of disease in the test fish (Brauhn and Schoettger, 1975).

To avoid the possibility of a build-up of metabolic or degradation products, the depletion of dissolved oxygen, the adsorption of the test substance to sediments and the walls of the test system, and to ensure an adequate mixing of the test solution, a minimum flow rate equivalent to six tank volume changes a day is specified. This flow rate appears to be a practical flow rate based upon past experience of successful use and satisfies Sprague's (1969) recommendation for a 90% replacement time of 8-12 hours.

c. Loading

The grams of organism per liter of solution in the test chambers should not be so high that it affects the results of the test. The most important parameters affected by overloading are the dissolved oxygen concentration, the waste metabolite accumulation, the concentration of the test substance, and stress to the test organisms due to crowding. Hence, loading should be limited to assure that: 1) the concentration of dissolved oxygen and test substance do not decrease below acceptable levels; 2) that above acceptable levels; and 3) that organisms are not stressed due to crowding.

The number of fish that can be placed in a given test chamber depends upon the test solution volume and the rate of flow. The upper limits are 2 grams of fish per liter of test solution in the test chamber at any given time, and 0.1 grams of fish per liter of test solution passing through the test chamber in 24 hours.

It is felt that these loading requirements can be easily and practically satisfied by all testing facilities without requiring modification to present equipment because of the

relatively small size and weight of the embryos and newly hatched larvae and developing juveniles of the recommended test species.

The levels of dissolved oxygen and ammonia cited in the test guideline are recommended for the health of the fish. The 20 percent limit on the uptake of test substance by the fish is recommended in order to avoid a reduction in the concentration of test substance to which the fish are exposed, such that the results become unsuitable for the purposes for which the test is run.

d. Temperature

Test temperature is dependent upon the species selected, but should not deviate instantaneously by more than 1.5°C from the selected temperature during the test period. The effects of sudden temperature changes on fish may range from death to temporary impairment of physiological function, depending upon the magnitude of the temperature change, the tolerance of the species to temperature fluctuations and the circumstances and duration of the exposure. Differences between the temperature at which fish embryos are obtained (i.e. culture or external shipments) and the temperature at which toxicity tests are conducted could have an adverse effect on the developing embryos. Therefore, it is desirable to obtain embryos at temperatures as close as possible to the temperatures at which the test will be conducted, and to acclimate embryos as specified. The test temperatures selected are: fathead minnow - 25°C (Brungs 1971a, USEPA 1972, Brauhn and Schoettger 1975, Brungs and Jones 1977); sheepshead minnow - 30°C (Schimmel and Hansen 1974, Hansen and Parrish 1977, USEPA 1978); and brook and

rainbow trout - 10°C for embryos and 12°C for alevins and fry (McCormick et al. 1972, Hokanson et al. 1973, Brungs and Jones 1977). These temperatures are generally accepted as suitable for the fish species indicated and they are the ones most frequently cited for use in conducting early life stage toxicity tests and for culturing the species selected. For example, Thatcher et al. (1976) studied the effects of temperature and chlorine toxicity on juvenile brook trout and concluded that 10°C to 15°C is perhaps the optimum temperature range for all the life promoting functions of this age of brook trout. This would obviously seem to be the range under which they would be least susceptible to temperature stress. Supporting this idea are the findings of McCormick et al. (1972) that indicate the suitable range of temperature for young brook trout growth and survival was from 9.8 to 15.4°C. In addition, the USEPA (1972b) recommended bioassay procedures for brook trout indicate that 15°C is the maximum desirable temperature for such studies.

Selection of temperatures which are "optimal" for growth, development and survival is necessary in order to develop data which accurately reflects the toxicity of a test substance. This rationale applies to all other test parameters such as dissolved oxygen, etc., which could stress the test organism and thereby skew test results. Unfortunately, a definitive data base is lacking to determine what temperature is "optimal" for each test species.

Temperatures were selected based upon available information relative to the culturing of each species, and

temperatures which have been successfully and widely used in toxicity tests. Extensive studies have not been reported in the literature which identify the optimal temperature at which early life stage toxicity tests with each test species should be run. There is, however, evidence that suggests the need to standardize the test temperatures. A number of investigators have reported toxicity data for several substances which indicate that the toxicity of those materials varies with temperature (L.L. Smith et al. 1978, Brown et al. 1967, Tucker and Leitzke 1979, Alexander and McClarke 1978). Therefore, even though data are lacking to unequivocally support selection of an optimal test temperature, it is recommended that early life stage toxicity tests be conducted at the test temperatures specified in the test guidelines. This is particularly necessary for comparative purposes.

Temperature is normally maintained by preheating and aerating the dilution water or by placing the test aquaria in temperature controlled water baths (Syrett and Dawson 1972, McCormick and Syrett 1970, Bahner et al. 1975). It should be noted that the latter method is not common practice for flow-through toxicity tests.

e. Light

Light is recognized as an important environmental variable by most organizations concerned with the development of uniform testing methods for aquatic toxicology. Yet, few studies on the effects of a light regime or light intensity on toxicity have been investigated and reported. Toxicity tests with pulp mill effluents did not demonstrate a significant variation in toxicity

associated with light-dark photoperiods of 8-16 hours and 16-8 hours (McLeay and Gordon 1978). However, in another study using Daphnia (Crosby et al. 1966), the photoperiod was shown to have an effect on the toxicity test results.

Although there are insufficient data on which to base the selection of a photoperiod to be used in fish toxicity tests, it is generally recognized that a standard photoperiod should be employed. This serves to minimize the influence of variations of light conditions on test data, as does standardization of other test conditions (i.e. temperature, dissolved oxygen, etc). The source material cited in Section II.C.1: Test Species, indicates that peak population levels and spawning activity (with the exception of trout) occur in the warmer months during which the photoperiods are naturally the longest. Thus, for the minnow species, longer photoperiods appear to correspond with natural conditions which are optimal for growth, development and survival. Standard Methods for the Examination of Water and Wastewater (American Public Health Association, American Water Works Association, Water Pollution Control Federation 1975) states that during any test exposure to light should be based upon what is normal for and required by the species, and that different light intensities are required for different organisms and life stages. One example cited in Standard Methods is the requirement of darkness (or very low light intensity) for trout eggs.

The photoperiods recommended by the ASTM (1980) for conducting early life stage toxicity tests with fishes are: brook trout 12-14 hours light; sheepshead minnow 16

hours light; and fathead minnow 16 hours light. A transition period of 15 to 30 minutes between light and dark is recommended (ASTM 1980) in order to avoid any possible variation in test results attributable to sudden light-dark changes. Apparatus which can be incorporated into indoor lighting systems to provide a programmed photoperiod can be designed according to Drummond and Dawson (1970) or by Wickham et al. (1971). Durotest vitalite (optima F8) lamps and wide spectrum Grow-lux fluorescent tubes are recommended, based upon past use.

D. Reporting

The reporting requirements specified in the test guidelines are considered essential to complete a thorough and proper evaluation of the test results. The required information is deemed necessary by EPA to: (1) establish that the test was conducted according to specifications; (2) evaluate those conditions and procedures that could affect the results of the test; and (3) supply sufficient information to accurately interpret results, which include independent analysis of statistics and conclusions. Due to the inherent flexibility of the test guidelines, reporting requirements are necessary to ascertain the conditions, parameters and observations germane to the performance of the test.

Additional information may be needed when a concern exists relative to the results or validity of the test. Therefore, the location of the raw data storage is needed in order that additional information can be located expeditiously, if necessary, for a detailed evaluation or for enforcement purposes. It is recognized that some

chemical companies maintain possession of test data, while other request the test facility to keep such records.

III. Economic Aspects

Five laboratories were surveyed to obtain estimated costs for performing the early life stage toxicity test outlined in the test guideline. A price range of $9,000 to $40,000 with a "best estimate" of $15,608 was reported. An additional cost estimate was made by separating the guideline into components and estimating the cost of each component, including direct labor cost, overhead cost, other direct costs, general and administrative costs, and profit or fee. The protocol best estimate of cost for the minnow species was $11,348, with an estimated range of $5,674 to $17,022 based on ± 50 percent of the best estimate. The protocol best estimate of cost for the trout species was $14,354, with an estimated range of $7,177 to $21,531 based on ± 50 percent of the best estimate. The protocol estimate for the mean of both species was $12,851 with an estimate range of $6,426 to $19,277.

The test guidelines described for minnows and trout have basic differences which result in different cost estimates. For example, the test with minnows requires a post-hatch exposure time of 28 days, while the test with trout requires a post-hatch exposure time of 60 days. This fundamental difference results in a higher cost in the trout test based on increases in animal care, observation intervals and analytical monitoring. Various other factors can affect the cost estimates, including the nature of the chemical, overhead rates, use of outside consultants and the degree of effects manifestation. For example, if a majority

of test organisms exhibited severe manifestations of toxicity, additional photography and detailed observations with possible pathology would be required. The nature and number of analytical assays is also a major cost factor.

The above cost estimates were made assuming that all the requirements of Good Laboratory Practice Standards, as specified in section (d) of the early life stage toxicity test guideline, and related considerations are contained in the cost analysis report for the ecotoxicity standards by Enviro Control, Inc. (1980).

In a cost analysis of subpart E, Hazard Evaluation: Wildlife and Aquatic Organisms, of the pesticide guidelines, several contract and captive industry laboratories were surveyed in 1978 and again in 1980 to determine cost estimates for testing (USEPA 1980a). The cited costs did not differentiate between species, however, the unit cost estimate for the fish embryo-larvae (early life stage) test was $11,500, and can assumed to be for fathead minnows.

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EG-12
August, 1982

SEED GERMINATION/ROOT ELOGATION TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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SEED GERMINATION/ROOT ELONGATION TEST

(a) Purpose. The guideline in this section is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (PUB.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes test procedures and conditions using seed of commercially important terrestrial plants to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (USEPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in Section 3 of the Toxic Substances Control Act (TSCA) and the definitions in Part 792--Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this guideline:

(1) "ECX" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Embryo" means the young sporophytic plant before the start of germination.

(3) "Germination" means the resumption of active growth by

an embryo. The primary root should attain a length of 5mm for the seed to be counted as having germinated.

(4) "Hypocotyl" means that portion of the axis of an embryo or seedling situated between the cotyledons (seed leaves) and the radicle.

(5) "Radicle" means that portion of the plant embryo which develops into the primary root.

(6) "Test solution" means the test chemical and the dilution water in which the test chemical is dissolved or suspended.

(c) Test procedures--(1) Summary of the test. (i) Seed should be separated into appropriate size classes, and that size class containing the most seed used exclusively for the test. Fresh test solutions should be added to petri dishes that have been completely filled with either precleaned quartz sand, 200 micron glass beads, or other inert material. The seed should then be positioned on the substrate allowing adequate room for anticipated growth. It is recommended that the radicle end of the seed be aligned in the direction of this growth. Petri dish lids should be used to hold the seed in place, and the dishes sealed with tape. For those chemicals that are insoluble in water and that should be sorbed to the substrate, deionized or glass-distilled water should be added to the substrate prior to positioning the seed.

(ii) The dishes should be placed in a seed germinator or other growth facility at a slight angle to facilitate linear root growth. Seed should be incubated in the dark until at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long.

(iii) The number of seed that germinate should be counted, and root lengths measured. Concentration response curves, EC 10's, and EC 50's for seed germination and root elongation should be determined and reported for each of the species tested.

(2) Chemical application. (i) Test chemicals that are soluble in water should be dissolved in deionized or glass distilled water and added to the substrate in the petri dishes at the start of the test.

(ii) Test chemicals that are insoluble in water but which can be placed in aqueous suspension with a carrier should be suspended in deionized or glass-distilled water with the carrier and then added to the petri dishes. The carrier should be soluble in water, relatively non-toxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol and others have extensively been used in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants.

Tests of the carrier effect should be included in the test experimental design and conducted simultaneously as controls.

(iii) Water-insoluble chemicals for which no non-toxic water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution and substrate should be placed in a rotary vacuum apparatus, and evaporated, leaving a uniform coating of test chemical on the substrate. A weighed portion of the substrate should be extracted with the same organic solvent and the chemical assayed before the containers are filled. Solvent controls should be included in the experimental design and tested simultaneously. Deionized or glass distilled water should be added to the treated substrate prior to positioning the seed on the substrate.

(3) Range-Finding Test. (i) A range-finding test should be conducted to establish (A) if definitive testing is necessary and (B) test solution concentrations for the definitive test.

(ii) The seed should be exposed to a chemical concentration series (e.g., 0.01, 0.1, 1.0, 10, 100, and 1,000 mg/l). The lowest concentration in the series, exclusive of controls, should be at the chemical's detection limit. The upper concentration, for water soluble compounds, should be the saturation concentration.

(iii) The test consists of one run for each of the recommended plant species or selected alternates. A minimum of 15 seed per species should be exposed to each chemical concentration and control. The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. The exposure period may be shortened if data suitable to establish the test solution concentration series for the definitive test can be obtained in less time and if the definitive test is to be conducted. No replicates are required; and nominal concentrations of the chemical are acceptable unless definitive testing is not required as specified below.

(iv) Definitive testing is not necessary if the highest chemical concentration tested results in less than a 50 percent inhibition of germination or reduction in root growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent inhibition of germination or reduction in growth.

(v) Graphical analysis of the range-finding data facilitates selection of chemical concentrations for the definitive test.

(4) Definitive test. (i) The purpose of the definitive test is to determine the concentration-response curves, the EC 10's, and EC 50's for seed germination and root elongation for

each species tested, with the minimum amount of testing beyond the range-finding test.

(ii) The seed of each species tested should be exposed to at least 6 concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2,4,8,16,32 and 64 mg/l). The concentration ranges should be selected to determine the concentration response curves between the EC 10 and EC 50 for both germination and root elongation. Test solutions or substrate extracts should be analyzed to determine chemical concentration prior to use. Selection of seed from the size class lot to be exposed to each test concentration should be unbiased.

(iii) At least three replicates, each with at least 10 seed per species should be tested for each concentration and control.

(iv) Every test should include controls consisting of the same dilution water, conditions, procedures and seed from the same lot used in the exposure group, except that none of the chemical is added. If a carrier (solvent) is needed to suspend or disperse the chemical, a separate carrier control should also be used.

(v) The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. When both conditions are satisfied, the mean

number of seed germinating and mean root length per treatment (and control) can be determined. If the test chemical concentration series does not bracket the EC10 through EC50 for both germination and root elongation, the test should be repeated (at a higher or lower concentration series). Concentration response curves, EC10's and EC50's for germination and root elongation should be determined for eace species tested and reported along with their 95 percent confidence limits.

(vi) Any abnormal seedling development or appearance such as lesions, enhanced root growth (measured), discoloration, swelling, loss of turgor, etc., should also be reported.

(vii) A randomized complete block design is recommended for the definitive test with blocks delineated within the seed germinator or growth chamber. If, for any reason, blocking is not feasible total randomization within chambers is acceptable.

(viii) Temperature in the germination facility should be recorded hourly. The pH of the test solutions should be recorded at the initiation of the definitive test.

(5) [Reserved]

(6) Analytical measurements--(i) Test chemical. Stock solutions should be diluted with glass distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these

solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(ii) Numerical. The number of seeds that germinate shall be counted and root lengths measured for each definitive test species. All root elongation measurements for a given species should be made sequentially before proceeding to the next species. Root length should be measured from the transition point between the hypocotyl and root to the tip of the root. Means and standard deviations should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves.

(d) Test conditions--(1) Test species. (i) Test plants recommended for use include:

Lycopersicon esculentum (tomato)

Cucumis sativus (cucumber)

Lactuca sativa (lettuce)

Glycine max (soybean)

Brassica oleracea (cabbage)

Avena sativa (oat)

Lolium perenne (perennial ryegrass)

Allium cepa (common onion)
Daucus carota (carrot)

Zea mays (corn)

(ii) Other species of economic or ecological importance to the region of impact, may also be appropriate for testing. A minimum of ten species should be tested.

(iii) Information on seed lot, the seed year or growing season collected, and germination percentage should be provided by the supplier of the seed. Only untreated seed (not treated with fungicides, repellants, etc.) taken from the same lot, and year or season of collection should be used in a given test. In addition, all seed of a species used in a test should be from the size class which contains the most seed. Damaged seed should be discarded. Standard seed dockage sieves should be used to size seed.

(2) Facilities--(i) Apparatus. (A) A seed germinator, or other controlled environment chamber capable of maintaining a uniform testing temperature of 25 ± 1°C is required. In addition, the facilities should include work areas for sizing, counting, and exposing seed for root measurement. If possible, these areas should be isolated from other activities. A fume hood may be needed when testing substances potentially hazardous

to human health. Apparatus for distilling and deionizing water are needed unless reagent grade water is used. Refrigeration facilities to hold the seed in cold storage (5°C) in moisture-proof containers at seed moisture contents of less than 10 percent are also needed.

(B) Disposal facilities should be adequate to accomodate spent glassware, sand, beads, and test solutions at the end of each run and any bench covering, lab clothing, or other contaminated materials.

(ii) Containers and support media. A minimum of 210 petri dishes and sufficient sand or glass beads, or other inert substrate to fill them are needed. Large (200 mm) glass petri dishes are recommended. Perlite, vermiculite, or native soils, should not be used as substrates.

(iii) Cleaning and sterilization. (A) All glassware and the substrate should be cleaned following standard good laboratory practice before each test. The substrate should be washed in half strength concentrated nitric acid and rinsed with a mild base followed by washes of glass-distilled or deionized water. The pH of the washed substrate should be near neutral. If the glass beads are to be reused, they should be heated to 100°C for 8-12 hours prior to acid washing. A dichromate solution should not be used for cleaning beads or petri dishes.

The sand and plastic petri dishes should not be reused.

(B) If fungal or other microbial contamination interferes with seed germination such that germination is less than 65 percent in the controls, glassware should be sterilized and/or the seed surface sterilized prior to use, e.g., the seed may be soaked for 10 minutes in a 10 percent sodium hypochlorite solution, then rinsed and soaked for one hour in glass-distilled water.

(3) Test parameters. Environmental conditions should be controlled to maintain incubation temperature at 25± 1°C in complete darkness. If species other than the ten recommended for use are tested, incubation conditions may have to be adjusted to meet germination and root length criteria in the controls.

(e) Reporting. The sponsor should submit to the USEPA all data developed during the test that are suggestive or predictive of phytotoxicity. In addition to the general reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the following should be reported:

(1) Information on the source and history of the seed, germination percentage reported by the supplier, and the seed size class used for testing.

(2) The number of seed of each species per treatment, the number of replicates, carriers, incubation conditions, and seed sterilization procedures.

(3) The concentration of the chemical added to each treatment dish and its pH (pH is optional).

(4) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1,000 mg/l) had no effect on the test species, report the results by species and concentration and a statement that the chemical is of minimum phytotoxic concern.

(5) If the range-finding test showed greater than 50 percent inhibition of germination or root elongation at a test concentration at the analytical detection limit, the results by species and concentration and a statement that the chemical is phytotoxic below the analytical detection limit.

(6) For each species included in the definitive test, means and standard deviations for germination and root length in each treatment. In addition, concentration response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and EC10's and EC50's identified.

(7) Methods and data records of all chemical and numerical analyses including method validation and reagent blanks.

(8) The data records of the incubation temperature. germination counts, and root length measurements.

ES-9
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

SEED GERMINATION/ROOT ELONGATION TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TECHNICAL SUPPORT DOCUMENT FOR SEED GERMINATION/ROOT ELONGATION TOXICITY TEST

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-12 which uses the seeds of various plant species to evaluate the toxicity of chemical substances on seed germination/root elongation. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. General

Chemicals may influence seedling vigor, a characteristic of increasing importance with increased mechanization in agriculture. Some crop plants (e.g., lettuce) with intense culture practices require high germination rates and vigorous growth. A single seed should germinate and establish a plant (planting to stand), and all plants should reach maturity simultaneously for once-over machine harvesting (Pollock and Roos 1972). Seedling establishment in forests also depends on vigorous root growth to survive environmental stresses and competition with other plants for light, water, and nutrients. In general, chemicals that reduce or delay germination and retard maturity of crops typically result in economic loss.

In agricultural land with other stresses (e.g., drought, salinity), the additional presence of chemicals which are toxic may be the limiting factor for production of crops on that land. In addition, chemicals may enter human food chains through processes associated with soil/plant interactions, uptake, translocation, and accumulation in food and forage crops. In natural systems, affected species are less competitive and with selection for tolerant species result in altered species diversity, density, and frequency of occurrence.

Toxic substances may also cause widely varying and significant effects on plant community dynamics. Many chemicals affect plants selectively, with some species sensitive to and others tolerant of the same chemical and dose. This selectivity may directly affect the successional replacement of one plant species by another, either by hastening the departure of an early successional species or by inhibiting the establishment of a later- stage successional species (Whittaker 1970). Succession also may be indirectly affected by inhibition of soil organisms. Toxic materials may produce high species diversity in some communities while reducing diversity in others (Brown 1978). Where plant growth and soil organisms are completely inhibited, soil degradation, instability, and eventually erosion, may result.

Seed germination and root elongation were selected to measure phytotoxicity for the following reasons:

- Seed germination is a critical stage in plant development. It marks the transfer from a period of dormancy to one of active growth and high

- metabolic activity. This process involves rapid cell division, expansion, and formation of the essential structures of a normal plant (Berlyn 1972).
- Phytotoxicity, as demonstrated by the inhibition of seed germination and root elongation, has been used in determining selective toxicities of herbicides (Behrens 1970, Horowitz 1976, Santelmann 1972), screening plants for heavy metal tolerances (Imai and Siegel 1973, Konzak et al. 1976, Siegel 1977, Whalley et al. 1974, Wilkins 1957), determining salinity tolerance (Durrant et al. 1974, Neiman and Poulsen 1971), evaluating chemicals for toxic effects (Hikino 1978, Rubinstein et al. 1975), and studying allelopathic substances (Asplund 1969, Bode 1958, Garb 1961, Muller 1965).
- Seed germination and root elongation show a reproducible response in proportion to the chemical concentration tested. In the literature pertaining to herbicide, heavy metal, and toxic chemical effects on plants, root elongation appears to give a more sensitive response than does seed germination and is the preferred test endpoint. Few herbicidal bioassays are based solely on the lethal concentration required to inhibit seed germination. More often, bioassays are based on sublethal concentrations that inhibit root elongation in which the response is dose-related (Horowitz 1976). The basis for root elongation tests can be seen in specific herbicidal studies.

- or example, the effects of picloram on germination and root elongation in safflower, radish, and barley were compared by Chang and Foy (1971). Germination was inhibited at 10^{-3M} while root elongation was inhibited at 10^{-5M} or 10^{-6M}. Similarly, the concentrations of potassium dichromate required to inhibit germination by 100 percent and 80 percent of control were 5×10^{-3M} and 1×10^{-3M}, respectively; while a concentration of 5×10^{-5M} showed a distinct inhibition of root elongation (Mukherji and Roy 1977).
- The test measures the inhibition of seed germination and the stimulation or inhibition of root growth. Both are easily observed or measured. In some situations it may not be clear whether germination or root elongation is inhibited, since the exact stage at which germination ends and growth begins is difficult to define. Some definitions of germination include the protrusion of the embryo through the seed coat. For the purpose of this guideline, seeds with at least a 5 mm protrusion of the embryo are counted as germinated. Requiring this growth allows consistent identification of successful germination. The slanted substrate technique, by enhancing linear root growth, facilitates root measurement.
- The test method is relatively rapid, simple, and inexpensive.

B. Test Procedures

1. Chemical Application

Chemicals have different physical and chemical properties which will influence the mode of application. Water-soluble chemicals will not present a problem but other chemicals will require different approaches. In this section we have attempted to provide general guidance by suggesting some approaches that have been used successfully in the past. There is a need for expert scientific judgement in the choice of solvent. The Agency recognizes the need to maintain flexibility, yet ensure that any effects are due to the test chemical and not the carrier. The suggested use of carrier controls to distinguish between test chemical and carrier effects is considered a standard laboratory practice.

2. Range-Finding Test

It is recommended that a range-finding test be conducted prior to the definitive test in those instances where no information is available on the phytotoxicity of the test chemical. This approach should reduce the risk of using an inappropriate concentration series in the definitive test. Under certain circumstances the range-finding test may preclude the need to conduct the definitive test. In order to minimize the cost and time required to obtain the requisite data, nominal concentrations are permitted, test duration may be shortened, replicates are not required, and other test procedures and conditions are relaxed.

If test results indicate that the chemical is non-toxic or very toxic to plants and if definitive testing is not conducted, it is necessary to ascertain that at least 65

percent of the control seed have germinated, the control root lengths have reached 2.0mm, and that the test was conducted at the specified incubation temperature. These verifications establish that the seed tested were viable and that the test was properly conducted.

In some situations there may be enough information available on toxicity to select the appropriate concentrations without a range-finding test. The range-finding test (or other available information) needs to be accurate enough to ensure that concentration levels in the definitive test are spaced above and below the EC10 and EC50 values for germination and root elongation. If the chemical has no measurable effect at the saturation concentration (at least 1000 mg/l), it is considered relatively non-toxic to seed germination/root elongation, and definitive testing for effects on these processes is deemed unnecessary. In all cases, the range-finding test is conducted to reduce the expense involved with having to repeat a definitive test because of inappropriate test chemical concentrations.

3. Definitive Test

The specific requirements of the definitive test are the analytical determinations of chemical concentrations, the unbiased selection of seed for each treatment, the use of controls, the assessment of test validity, and the recording, analysis, and presentation of data. These requirements assure that the chemical concentration - plant response relationship is accurately known, that chemical effects are not confounded by differential seed vigor, and that the relationships are clearly presented. Reporting the occurrence of such abnormal effects as lesions,

discoloration, or lack of turgor provides qualitative data that further assist the assessment of phytotoxicity.

The purpose of the definitive test is to determine the EC10, EC50, and concentration-response curves for seed germination and root elongation for each species tested with a minimum of testing beyond the range-finding test. It is probable that each of the species tested may have a different response curve for a given chemical based on the range-finding test and that more than six concentrations of a test substance in a geometric series may be needed to properly describe the dose-response relationships for all species being tested. By testing a minimum of six concentrations in a series per species, partial effects (lack of germination and/or reduced root length) will be probable and the dose response relationship will be better defined. The slope and shape of the dose-response curve will allow estimation of the effects of lower concentrations on the test plants.

The primary observations - number of seed per species per chemical dose which germinate, measurement of root growth, and determination of the actual chemical concentrations employed in the definitive test - are all needed to accurately describe the dose response curve from which the EC10 and EC50 are calculated.

The recommended experimental design is the randomized complete block. As discussed by Hammer and Urquhart (1979), it is essential that the investigator randomly assign petri dishes to treatments to assure that each sample of seed has the same chance of receiving any of the treatments (exposure level of test chemical). To account for variation within

the germination chamber and to increase the sensitivity for detecting treatment differences, small square blocks should be delineated in the germination chamber with randomization of treatments within blocks. Replication should occur over chambers (of the same type) as, in many cases, a within-chamber estimate of residual variance badly underestimates the between-chamber estimate (Hammer and Urquhart 1979). This means that differences between chambers are often greater than differences in growth and environmental conditions within chambers. In the event that blocking within chambers is impossible, total randomization is acceptable.

In order to substantiate that temperature was maintained within specified limits, it will be necessary to measure and record temperature throughout the test. Requisite instrumentation is readily available, easy to maintain, and should not increase complexity or costs of the test. Temperatures should be recorded hourly to prevent any severe fluctuations that might affect growth processes and/or chemical uptake.

4. Analytical Measurements

The actual chemical concentration used in the definitive test should be determined with the best available analytical precision. Analysis of stock solutions and test solutions just prior to use will minimize problems with storage (e.g., formation of degradation products, adsorption, transformation, etc.). Nominal concentra-tions are not adequate for the purposes of the definitive tests. If definitive testing is not required because the chemical elicits an insufficient response at the 1000 mg/l level in

the range-finding test, the concentration of chemical in the test solution should be determined to confirm the actual exposure level.

The pH of the test solution should be measured prior to testing to determine it if lies outside of the species' optimal range. While it is recognized that seeds of crop plants germinate over a broad range of hydrogen-ion concentrations and typically exhibit a pH optima for germination, this test guideline does not include pH adjustment for the following reasons: the use of acid or base to adjust pH may chemically alter the test chemical making it more or less toxic; the amount of acid or base needed to adjust the pH may vary from one test solution concentration to the next, and the effect the test chemical has on pH may indirectly affect the growth and development of the test plant. Therefore, the pH of each test solution should be determined and compared with the acceptable range for growth and development of the test species.

To reduce variability resulting from continual root elongation during the measurement period, it is recommended that measurements be made sequentially (by concentration) within a given species.

The data obtained in bioassays are usually expressed as standard response curves in which growth response of the test species is plotted against the concentration of the test chemical. The manner of expressing plant response varies considerably. For this guideline, plant growth responses are expressed as direct measurements of number of seed that germinate and root growth. The statistical analysis (goodness-of-fit determination) facilitates

accurate calculations of EC10 and EC50 as well as providing confidence limits for the concentration- response curves.

C. Test Conditions

1. Test Species

a. Selection

The ten terrestrial plant species recommended for the seed germination/root elongation, early seedling growth, and plant uptake test guidelines are as follows:

Lycopersicon esculentum (tomato)
Cucumis sativus (cucumber)
Lactuca sativa (lettuce)
Glycine max (soybean)
Brassica oleracea (cabbage)
Avena sativa (oat)
Lolium perenne (perennial ryegrass)
Allium cepa (common onion)
Daucus carota (carrot)
Zea mays (corn)

In addition, other species of economic or ecological importance to the region of impact may also be tested in lieu of these species.

These ten species have been selected for the following reasons:

- As food, forage, or ornamentals, they are economically important and constitute major cash crops.

- Their distribution, abundance, and taxonomic representation suggest broad coverage of the plant kingdom.
- They are also specified for phytotoxicity testing of pesticides (Subpart J, Pesticide Registration Guidelines). Additional justification for selection of these test species is provided in these guidelines (see FR 45(214): 72948-72978).
- They are sensitive to many toxic compounds and have been used to some degree in previous bioassays. Their use in herbicidal bioassays, heavy metal screening, salinity and mineral stress tests, and allelopathic studies indicates a sensitivity to a wide variety of stressors (Guenzi and McCalla 1966, Geronimo et al. 1973, Puerner and Seigel 1972, Wiedman and Appleby 1972, Reynolds 1978, Chang and Foy 1971).
- They are compatible with the environmental growth conditions and time constraints of the test method. Seed from the selected species germinate quickly and easily. Root growth is rapid and uniform. The seed contain no natural inhibitors and require no special pretreatment to germinate (such as soaking, chilling, prewashing, light, or scarification). Seed size is compatible with the test system; large enough to be easily manipulated with forceps but not so large to be space limiting in the petri dishes. All the seed grow rapidly under the prescribed environmental conditions and most have measurable root lengths within a one week period.

Other species may be substituted for any or all of these ten species when appropriate. For example, forest or desert species may constitute the population at greatest risk. If so, those of most value to man or of ecological dominance to the affected ecosystem should be selected. The rationale for selection of alternative species should be discussed with the Agency and/or supported in the report of findings.

No single plant will always be the most or least sensitive to all chemicals which may be tested. The use of different types of plants ensures that variations in plant response will be evident. In a seedling growth test, Hikino (1978) used concentrations of 0.01 to 1,000 ppm for eight chemicals. Rice, turnip, and soybean seed were placed in petri dishes with test chemical and agar medium and incubated in the dark. Root and shoot development were measured each day and at the end of the test period. Six of the eight chemicals inhibited root growth at 100 or 1,000 ppm for each species. The other two chemicals inhibited root growth in at least one of the species. It is important to note that none of the three species tested was consistently the "most sensitive". These results further support the requirements of testing several species.

The definitive test requires that seed of the same size class be used throughout the test. Germination rate and percentage, seedling vigor, initial growth rate, and sensitivity to chemical stress are related to seed size. For example, Anderson (1969) found a significant correlation between soybean seed size and sensivitity to the herbicide atrazine, with the smaller seed being more sensitive than the larger seed. Also, the larger or heavier the seed, the

greater the percent germination and the more vigorous the seedling (Pollock and Roos 1972, Smith et al. 1973, Sharples 1970, Whalley et al. 1966, Carleton and Cooper 1972). The differential response to chemical stress suggests that seed of the smallest size class be used for testing to take advantage of the increased sensitivity. However, as use of seed from the smallest size class carries with it a reduction in viability and a probable increase in variability, it is recommended that larger seed be used. By selecting seed from the size class containing the most seed, assurance is provided that sufficient seed are available for testing, that the percent viability is sufficiently high for valid testing, and that seed sensitivity to the test chemical is representative of the species.

Information provided by the seed supplier provides additional assurance that the seed are viable. Use of seed produced during one growing season minimizes problems associated with differential viability between lots. By using untreated seed, possibilities of confounding test results with fungicides, repellants, etc., are eliminated.

2. Facilities

a. Apparatus

The test requires a germination chamber or temperature-controlled enclosure capable of maintaining a uniform temperature of 25° ± 1°C. Other facilities typically needed for conducting seed germination/root elongation tests include standard laboratory glassware, petri dishes, work areas to clean and prepare equipment and to measure chemical concentrations and plant responses, refrigeration to hold the seed until needed for testing, and proper disposal

facilities. Without these facilities, the testing cannot be adequately conducted.

b. Containers and Support Substrate

Petri dishes should be composed of inert materials such as borosilicate glass in order to minimize reaction of the test chemical and/or carrier with the container. The growth support substrate should also be composed of inert material for these same reasons. Quartz sand and glass beads are suitable inert materials and minimally sorb substances, ensuring that the chemical will be maximally available to the inbibing seed and developing embryo. The chemical would not be as readily available if it were sorbed to the substrate or container. Sand or glass beads are used, rather than vermiculite, perlite, or soil, to avoid complications associated with variable physical and chemical properties and microbial populations indigenous to native soils. Native soils are undesirable because of the varying clay, sand, and humus components, the types and proportions of which vary within the same soil type. Microbial populations also vary between soil types. These variables alter moisture-holding capacity, chemical-binding capacity, aeration, and nutrient and trace element content (Audus 1964, Beetsman et al. 1969, Beall and Nash 1969). In addition to the variations in these physical factors, there will also be variation in such chemical properties as pH and redox potential. Because of the impossibility of controlling physical and chemical properties of native soils, inert material is required to support the plants with the only variables being the presence and concentration of test chemical. The purpose of using glass beads or sand

instead of native soils is not to make test results more directly applicable to natural systems, rather it is to eliminate sources of variation in the test.

c. Cleaning and Sterilization

Standard good laboratory practices are recommended to remove dust, dirt, other debris, and organic and inorganic residues from the test containers and support media which might confound test results. Residues could enter the test solution and be taken up by the developing embryos, affecting their growth and/or their metabolic activity, causing misleading plant response. Dichromate solution should not be used for washing glassware or the glass beads since dichromate inhibits germination and reduces root elongation (Mukherji and Roy 1977). Sand or plastic petri dishes should not be reused to insure against residue carry-over.

Since untreated seed are used for all testing, fungal contamination during testing may reduce germination and root growth. If fungal contamination is a problem, the testing laboratory should evaluate glassware cleaning procedures, laboratory ventilation, and other possible contributory sources and correct them first. If these actions do not control the problem, then the seed sterilization procedure described in the test guideline or another appropriate technique should be utilized. The sterilization procedure selected, however, should not bias testing results.

3. Environmental Conditions

Controlled environmental conditions are necessary to maintain uniform growth and ensure reliable data. The essential environmental factors for germination are an

adequate supply of water, a suitable temperature, an adequate supply of oxygen, and in some species, light. Temperature is the one single variable that could most readily affect metabolic respiration and thereby growth rate and chemical effect. The temperature range suitable for germination is quite broad for most species. Within this range there is usually an optimal temperature at which the highest percentage of germination is attained in the shortest time. A single temperature was selected which was near the optimal range for germination and root elongation of all test species and commonly used in seed viability studies.

The seed of the selected species will germinate in continuous dark. Incubators, seed germinators, or controlled environment chambers that provide temperature control are available commercially. Without the need for controlled light intensity or photoperiod, a constant temperature is easily maintained at 25 $\pm$ 1°C and cost is reduced compared with costs of lighted chambers.

Both seed germination and root elongation are dependent upon an adequate supply of water. Adequate uniform moisture for germination and root elongation is provided by maintaining saturation of the growth substrate (quartz sand, or glass beads) with test solution in petri dishes. Sealing the petri dishes with tape or enclosing them in plastic bags reduces the rate of water loss.

Both seed germination and root elongation depend upon adequate oxygen. An adequate supply of air may not be present to allow normal growth and development in petri dishes if they are completely sealed. It may be necessary

to leave the petri dish lid partially unsealed in order to provide sufficient oxygen to support respiration.

D. Reporting

The sponsor should submit to the Agency all data developed during the test which are suggestive or predictive of phytotoxicity. In addition, information supplied with the seed and the size class of seed used for testing are required because these data have a bearing on the validity of the test. If testing specifications are followed, the sponsor should report that specified procedures were followed and present the results. If alternative procedures were used instead of those recommended in the test guideline, then the protocol used should be fully described and justified.

Test temperature, chemical concentrations, test data, concentration response curves, and statistical analyses should all be reported. The justification for this body of information is contained in this support document. If plant species other than the ten recommended were used, the rationale for the selection of the other species should be provided.

III. Economic Aspects

The Agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for performing an early seedling growth test according to this Guideline. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

The protocol estimate was $1,687. This estimate was prepared by identifying the major tasks needed to do a test and estimating the hours to accomplish each task.

Appropriate hourly rates were then applied to yield a total direct labor charge. An estimated average overhead rate of 115%, other direct costs of $200, a general and administrative rate of 10%, and a fee of 20% were then added to the direct labor charge to yield the final estimate.

Enviro Control estimated that differences in salaries, equipment, overhead costs and other factors between laboratories could result in as much as 50% variation from this estimate. Consequently, they estimated that test costs could range from $843 to $2,531.

The laboratory survey estimate was $950, the mean of the estimates received from two laboratories. The estimates ranged from $700 to $1,200 and were based on the costs to perform the test according to this Guideline.

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EG-13
August, 1982

EARLY SEEDLING GROWTH TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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EARLY SEEDLING GROWTH TEST

(a) Purpose. The guideline in this section is intended for use in developing data on the toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003, 14 U.S.C. 2601 et. seq.). This guideline prescribes tests using commercially important terrestrial plants to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (USEPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA), and Part 792--Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline.

(1) "EC X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Germination" means the resumption of active growth by an embryo.

(3) "Support media" meant the quartz sand or glass beads used to support the plant.

(c) Test Procedures--(1) Summary of the Test--(i) Root exposure. In preparation for the test, seeds are planted in the potting containers (or in cotton or glass-wool plugs supported in hydroponic solution) and after germination seedlings are thinned by pinching the stem at the support medium surface to the ten (10) most uniform seedlings per pot. This marks the start of the test and the time of first application of test chemical. Seedlings emerging after this time are also pinched off at the surface. Potting mixtures of sand or glass beads are subirrigated with nutrient solution. Chemicals are applied to the plants via nutrient solution or are adsorbed to the support media. Plants are harvested after 14 days and analyzed for growth.

(ii) Foliar exposure. The foliar exposure test is identical to the root exposure test except that chemicals are applied to plants by either spraying or dusting the foliage or by exposing the plants to gas in a fumigation chamber.

(2) Chemical application--(i) Root exposure. (A) Chemicals that are soluble in water should be dissolved in the nutrient solution just prior to the beginning of the test. Deionized or glass-distilled water should be used in making stock

solutions of the test chemical. Sufficient quantities of each concentration should be made up as needed to minimize storage time and disposal volume.

(B) Chemicals that are insoluble in water, but which can be suspended in an aqueous solution by a carrier, should be added, with the carrier, to the nutrient solution. The carrier should be soluble in water, relatively non-toxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol, and others have extensively been used in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants. Carrier controls should be included in the experimental design of the test and tested simultaneously.

(C) Water-insoluble chemicals for which no non-toxic, water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution should be mixed with the sand or glass beads which are then placed in a rotary vacuum apparatus and evaporated leaving a uniform coating of chemical on the sand or beads. A weighed portion of beads should be extracted with the same organic solvent and the chemical assayed before the potting containers are filled. Solvent controls should be included in the experimental design and tested

simultaneously.

(ii) Foliar exposure. (A) Water-soluble chemicals should be dissolved in deionized or glass-distilled water just prior to use. Sufficient quantities of each concentration should be made up as needed. These solutions should be applied daily (during the normal five-day work week). Plants should be placed in an exhaust hood and the chemical applied to the foliage. A plastic sleeve may be fitted over the top of the pot, and the foliage sprayed with specific quantities of test solution at known concentrations. The plastic sleeve, confining the chemical to plant and pot, facilitates expression of chemical dosage to quantity per pot area (i.e., ug/m^2). Shoots of control plants should be sprayed in an identical manner with deionized or distilled water. Alternatively, a miniature compressed-air sprayer mounted on a pendulum and equipped to automatically spray a plant positioned directly beneath the center of its arc of swing may be used.

(B) Water-insoluble chemicals, existing as solids, may be prepared for testing by grinding or other reduction to particles of <200 um diameter. Each day (during the normal five-day work week) plants should be placed in an exhaust hood, a plastic sleeve fitted over the top of the pot, and specific quantities of chemical sprinkled uniformly over the potted seedlings. Prior to

chemical application, plants should be misted with water to promote foliar retention of the chemical. Control plants should also be misted with deionized or distilled water at each treatment date and dusted with an inert material of the same particle size. Applications are expressed as quantity per unit pot area (i.e., ug/m^2).

(C) Chemicals existing in gaseous form at normal ambient temperatures and pressures can be generated as needed or stored under pressure. The bottled gas may be 100 percent chemical or may be mixed with an inert carrier, such as nitrogen, to known concentrations. Chemicals of controlled or measured concentrations should be metered into the exposure chamber, uniformly mixed about the plants, and exhausted through an outlet port.

(3) Range-finding test. (i) A range-finding test should be conducted to establish (A) if definitive testing is necessary and (B) the concentrations of test substance used in the definitive test for each species.

(ii) The recommended procedure is to expose newly germinated seedlings to a series of widely spaced concentrations of test chemical and assess effect as growth reduction. Seeds (approximately 30) should be planted directly in containers filled to within 2.5 cm of the top with quartz sand or glass

beads. If a hydroponic system is used, the seeds should be planted in plugs of cotton or glass wool supported at the top of the solution. When 50 percent of the seeds have germinated the seedlings should be thinned (by pinching) to the 10 most uniform per pot and exposed to a widely spaced concentration series (i.e., 0.01, 0.1, 1.0, 10, 100, 1,000 mg/l) of test chemical. The lowest concentration in the series, exclusive of controls, should be at the chemical's detection limit. The upper concentration, for water-soluble compounds, should be the saturation concentration. If the anticipated fate of the chemical is soil or soil water, and the mechanism of concern is root uptake, the chemical should be applied in nutrient solution to the root support media (or coated on sand or glass beads for non-water soluble chemicals). With a chemical whose anticipated mode of exposure to plants is surface deposition by atmospheric transport, or irrigation water, the appropriate testing method may be foliar application allowing subsequent movement into the rooting zone with watering. Effect is assessed as growth reduction.

(iii) Alternatively, the seed germination/root elongation test may be used to establish the appropriate concentration range for testing.

(iv) No replicates are required and nominal concentrations are acceptable unless definitive testing is not required.

(v) Definitive testing is not necessary if the highest chemical concentration tested results in less than a 50 percent reduction in growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent reduction in growth.

(4) Definitive test. (i) The purpose of the definitive test is to determine the concentration response curves and the EC 10's and EC 50's for each of the species tested with the minimum amount of testing beyond the range-finding test.

(ii) At least 5 concentrations of chemical, exclusive of controls, should be used in the definitive test. For each species tested the concentration range should be selected to define the concentration-response curve between the EC 10 and EC 90. Test chemicals should be added to the hydroponic or nutrient solution or coated on the support media for the root exposure test; or sprayed, dusted, or gassed directly on the foliage in the foliage exposure tests.

(iii) Control pots should be included in the experimental design and should be used in each run. In addition, a carrier control should also be used for those chemicals that need to be solubilized.

(iv) If plants are to be grown hydroponically, seeds should be planted in plugs of cotton or glass wool supported in the tops of the containers. When sand or glass beads are used, the recommended planting procedure is to fill the potting containers to within 2.5 cm of the top and to sow seeds directly on the support media. After 50 percent of the seeds have germinated, the seedlings should be thinned to the 10 most uniform per pot.

(v) Alternate planting methods may be required when the chemical is highly volatile. An impervious barrier of polyethylene film, a modification of the double pot method, a glass plate, or other appropriate apparatus should be used to prevent volatilization from the root zone. Seeds should be germinated in the dark at 25°C and seedlings with radicle lengths in the median range transplanted into the potting containers. The seedlings should be positioned such that their roots are exposed to the support media while the shoots pass through holes in the barrier. A ring of non-toxic, inert, pliable putty should be used to seal the holes around the stems. Control pots should be handled identically to the test pots except there is no exposure to the test chemical. This transplanting procedure, without the volatilization barrier, is also recommended when the test chemical is adsorbed to the support medium.

(vi) The test consists of one run for each of the recommended plant species or selected alternates. The duration of a run should be at least 14 days from the time that 50 percent of the seeds have germinated. For a particular chemical, a run is defined as exposure of the plant species to five concentrations of the chemical in a minimum of 3 replicate pots (10 plants per pot), with appropriate controls, followed by weight and height determinations and analysis.

(vii) All abnormalities (visible effects of the chemicals on plant growth and morphology including stunting of growth, discoloration, chlorosis and/or necrosis of the leaves, or morphological abnormalities) should be recorded. Observations plants should be made daily (during the normal five-day work week).

(viii) A randomized complete block design is recommended for this test with blocks delineated within the chambers or over greenhouse benches and randomization of treatment occurring within the blocks. If, because of very large pots, there exists inadequate space within chambers for blocking, total randomization within chambers is acceptable.

(ix) Irradiation measurements should be taken at the top of the plant canopy and the mean, plus a maximum and a minimum value, determined over the plant-growing area. These

measurements should be taken daily and should be taken at least at the start and finish of the test. If the test is conducted in a greenhouse facility, hourly measurements of irradiation should be recorded and presented as daily total irradiance plus representative hourly curves for clear sky conditions and cloudy days.

(x) Temperature and humidity should be measured daily at the top of the plant canopy during each light and dark period.

(xi) Measurements of carbon dioxide concentration should be made at the top of the plant canopy (of chamber- grown plants) on a "continuous basis".

(5) [Reserved]

(6) Analytical measurements--(i) Chemical. Stock solutions should be diluted with glass distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(ii) Numerical. Mass and length of roots, shoots, and entire plants (root and shoot) should be measured for the

definitive test. Means and standard deviations should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves.

(d) Test conditions--(1) Test Species--(i) Selection. (A) Test plants recommended for the definitive test include:

Lycopersicon esculentum (tomato)

Cucumis sativus (cucumber)

Lactuca sativa (lettuce)

Glycine max (soybean)

Brassica oleracea (cabbage)

Avena sativa (oat)

Lolium perenne (perennial ryegrass)

Allium cepa (common onion)

Daucus carota (carrot)

Zea mays (corn)

(B) Other species, of economic or ecologic importance to the region of impact, may also be appropriate and selected for testing.

(ii) Seed selection. Information on seed lot, the seed year or growing season collected and germination percentage should be provided by the source of the seed. Only untreated seed (not treated with fungicides, repellants, etc.) taken from the same

lot, and year or season of collection should be used in a given test. In addition, all seed of a species used in a test should be of the same size class; and that size class which contains the most seed should be selected and used in a given test. Any seed which is damaged should be discarded.

(2) Facilities--(i) Apparatus. (A) Greenhouses or environmental chambers should provide adequate environmental controls to meet the carbon dioxide, humidity, irradiation, photoperiod, and temperature specifications. Chambers should be designed to prevent escape of internal air into the external environment other than through appropriate filtering material or media to prevent contamination of the external environment with the test chemical.

(B) Laboratory facilities for chemical determinations should include non-porous floor covering, absorbant bench covering with non-porous backing, and adequate disposal facilities to accommodate plant nutrient, test and wash solutions containing test chemicals at the end of each run, and any bench covering, lab clothing, or other contaminated materials.

(ii) Containers and support media. For each run, 18 polyethylene pots sufficiently large to grow at least 10 plants up to 14 days, are required for each species. It is equally acceptable to use small, individual containers if plants are

grown in hydroponic solution. An additional 3 pots will be needed if a carrier control is needed. Potting containers used in each experiment should be of equal size and volume and possess the same configuration. When sand or glass beads are used, the potting containers should be filled to within 2.5 cm of their tops. Perlite, vermiculite, native soils, etc., should not be used for root support.

(iii) Cleaning and sterilization. (A) Potting and receiving containers, nutrient storage containers, and root support medium should be cleaned before use. All equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or prior use. A dichromate solution should not be used for cleaning beads or pots.

(B) Rooting media other than glass beads should be discarded at the end of the experiment. Disposal should conform to existing regulations.

(iv) Nutrient media. Half-strength modified Hoagland nutrient solution should be utilized as nutrient media for this test. When sand or glass beads are used as a support media, the potting containers should be filled with nutrient solution and drained periodically. An automated system design is recommended.

(3) Test parameters. Environmental conditions should be maintained as specified below:

(i) Carbon dioxide concentration at 350 ± 50 ppm.

(ii) Relative humidity should approach 70 ± 5 percent during light periods and 90 percent during dark periods.

(iii) Irradiation, measured at 1 meter from the source, at 350±50 uE/m^2 sec at 400-700 nm.

(iv) Photoperiods of 16 hours light and 8 hours darkness.

(v) Day/night temperatures at 25°/20° ± 3°C.

(e) Reporting. Reporting requirements of Part 792--Good Laboratory Practice Standards apply to this guideline. The following data should be reported for each of the species tested in tabular form:

(i) Concentration of chemical in nutrient solution and in the root support material when the chemical is soluble in water or solubilized with a carrier compound; or the concentration of carrier compound in nutrient solution when carrier is used; or the quantity of chemical per unit weight of root support material when it is coated on the material.

(ii) The quantity of chemical, the concentration at which it was applied, and the number of applications for those chemicals applied to the foliage.

(iii) Environmental conditions (day/night temperatures, relative humidity, light intensity, carbon dioxide concentration, and photoperiod).

(iv) Mass of above ground (shoot) and below ground (root) portion of each plant and mass of each whole plant (dry weight at 70°C).

(v) Length of shoot, root, and entire plant.

(vi) Visible effects of chemical, if any, on the intact plants.

(vii) Means and standard deviations for mass and length of roots, shoots, and entire plants in each treatment and control. In addition, concentration- response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and EC 10's and EC 50's identified.

ES-10
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

EARLY SEEDLING GROWTH TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TECHNICAL SUPPORT DOCUMENT FOR EARLY SEEDLING GROWTH TOXICITY TEST.

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-13 which uses various plant species to evaluate the toxicity of chemical substances on early seedling growth in plants. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. General

Chemicals may influence seedling vigor, a characteristic of increasing importance with increased mechanization in agriculture. Some crop plants (e.g., lettuce) with intense culture practices require high germination rates and vigorous growth. A single seed should germinate and establish a plant (planting to stand), and all plants should reach maturity simultaneously for once-over machine harvesting (Pollock and Roos 1972). Seedling establish-ment in forests also depends on vigorous root growth to survive environmental stresses and competition with other plants for light, water and nutrients. In general, chemicals that reduce or delay germination and retard maturity of crops typically result in economic loss.

In agricultural land with other stresses (e.g., drought, salinity), the additional presence of chemicals which are toxic may be the limiting factor for production of crops on that land. In addition, chemicals may enter human food chains through processes associated with soil/plant interactions, uptake, translocation, and accumulation in food and forage crops. In natural systems, affected species are less competitive and with selection for tolerant species result in altered species diversity, density, and frequency of occurrence.

Toxic substances may also cause widely varying and significant effects on plant community dynamics. Many chemicals affect plants selectively, with some species sensitive to and others tolerant of the same chemical and dose. This selectivity may directly affect the successional replacement of one plant species by another, either by hastening the departure of an early successional species or by inhibiting the establishment of a later stage successional species (Whittaker 1970). Succession also may be indirectly affected by inhibition of soil organisms. Toxic materials may produce high species diversity in some communities while reducing diversity in others (Brown 1978). Where plant growth and soil organisms are completely inhibited, soil degradation, instability and eventually erosion may result.

Early seedling growth was selected to measure phytotoxicity for the following reasons:

- The early seedling growth phase is a critical stage in plant development. Occurring immediately after germination, it constitutes a period of rapid

vegetative growth which is dependent upon such physiological processes as photosynthesis, water and nutrient uptake, and metabolic conversions of photosynthates to structural products (such as cell walls). Chemical interference with any of these physiological processes would be evident as reduced growth. The only physiological processes not included in this test are the germination of the seed and the reproductive phases (flowering, pollination, fruit set).

- o The test measures the inhibition of shoot and root growth. Both are easily observed and measured as length and as mass.
- o The test method is relatively rapid, simple, and inexpensive.

The test design is a composite of prior experiments that investigate the effects of heavy metals, pesticides, or organic chemicals on plants (Beall and Nash 1971, Cole et al. 1976, Chou et al. 1978, Fuhr and Mittelstaedt 1980, Kelly et al. 1980, Wickliff et al. 1980). The test is rapid, simple, and has been successfully used to measure chemical effects on seedling growth.

B. Test Procedures

1. Chemical Application

Chemicals have different physical and chemical properties which will influence the mode of application. Water-soluble chemicals will not present a problem but other chemicals will require different approaches. In this section we have attempted to provide general guidance by suggesting some approaches that have been used successfully

in the past. There is a need for expert scientific judgement in the choice of solvent. The Agency recognizes the need to maintain flexibility, yet insure that any effects are due to the test chemical and not the carrier. The suggested use of carrier controls to distinguish between test chemical and carrier effects is considered a standard laboratory practice.

Water soluble chemicals are easiest to apply, since roots may absorb chemicals directly from hydroponic or nutrient solutions and many air-borne chemicals arrive in precipitation. Water insoluble chemicals require other procedures to ensure contact between the test chemical and the root and shoot systems. Appropriate controls are necessary and are designed to reduce to the extent possible the number of variables. One means of incorporating a water insoluble chemical into the hydroponic solution or the nutrient medium is to solubilize it in the solution with a surfactant or carrier compound. When a carrier is used, a carrier control is included in the procedure to check carrier's effects on plant growth. A minimum quantity of carrier should be used to minimize carrier effects.

Foliar applications of powders or sprays should be done under a hood to ensure worker safety and to minimize growth chamber contamination. Control plants should also be moved from chambers to the hood area and sprayed with distilled, deionized water whenever the chemical is applied to other plants by spraying because plants are highly sensitive to mechanical stresses such as handling (Wheeler and Salisbury 1979).

Attention to maintenance of environmental conditions during applications of gasses is essential to the success of the test. Brenann and Leone (1968) observed that plants exposed to identical concentrations of pollutants at different gas flow rates exhibited different toxicity symptoms. These observed variations are understandable when considered in terms of the effect of environmental physics on mass transport to the leaves and on the physiological condition of the plants. In order that each plant in the gas exposure chamber receive the same dosage, chamber design should provide a spatially uniform test chemical distribution. Movement of gaseous substances to the leaf surfaces is dependent upon air turbulance around the leaves reducing the boundary layer thickness (Fowler and Unsworth 1974, O'Dell et al. 1977). Some substances may impact or condense on the surface of the plants (Fowler and Unsworth 1974) while others may diffuse through stomatal pores or across the cuticle to the leaf's interior (Black and Unsworth 1979, Rich et al. 1970). The cuticle provides an effective barrier for gaseous substances and several investigators have shown chemical absorption to be regulated by the same factors that control stomatal opening including light, temperature, humidity, carbon dioxide concentration, and the plant's water status (Bennett et al. 1973, Black and Unsworth 1979, Rich et al. 1970, Winner and Mooney 1980). Specified environmental conditions allow for normal stomatal response and plant growth. These conditions should be maintained during plant exposure to chemicals. The exposure chamber designed by Rogers et al. (1977) meets the environmental criteria and the criteria of uniform gas

distribution within the chamber.

The growth medium control provides a baseline check of the effects of hydroponic solution, potting material, nutrient solution, and the testing environment on plant growth during each test. This control is simply the plant growing in the hydroponic solution or root support material and maintained by the nutrient solution without test chemical, solvent, or carrier. This control is required in each run regardless of whether or not the chemical is soluble in water. It is the only control used when the chemical is water soluble and applied directly to the root support media or when it is applied as foliar dust or spray.

A second control is also recommended whenever a carrier (solubilizer, or non-aqueous solvent) should be used in order to maximize contact between the chemical and the plant. The solvent or carrier control is used to check carrier effects on plant physiology and growth.

2. Range-Finding Test

It is recommended that a range-finding test be conducted prior to the definitive test in those instances where no information is available on the phytotoxicity of the test chemical. This approach should reduce the risk of using an inappropriate concentration series in the definitive test. Under certain circumstances the range-finding test may preclude the need to conduct the definitive test. In order to minimize the cost and time required to obtain the requisite data, nominal concentrations are permitted, test duration may be shortened, replicates are not required and other test procedures and conditions are relaxed.

The concentration range for the selected definitive test is normally based on the results of a range-finding test that is conducted to reduce the time and expense involved in having to repeat definitive tests. Results of previously conducted plant effects tests can be used to this end. For instance, the seed germination/root elongation test can be used to establish the appropriate chemical concentration range for the early seedling growth test. Concentrations which inhibit seed germination and/or elongation are likely to be inhibitory to many other metabolic activities of seedlings and maturing plants. However, for those chemicals that inhibit photosynthesis, the seed germination/root elongation test may not provide a suitable range-finding test.

3. Definitive Test

The specific requirements of the definitive test are the analytical determinations of chemical concentrations, the selection of uniformly sized seedlings, the unbiased selection of seedlings for each treatment, the use of controls, and the recording, analysis, and presentation of data. These requirements assure that the chemical concentration-plant response relationship is accurately known, that chemical effects are not confounded by differential seedling vigor, and that the relationships are clearly presented. Reporting the occurrence of such abnormalities as stunting, discoloration, chlorosis or necrosis provides qualitative data that further assist the assessment of phytotoxicity.

The purpose of the definitive test is to determine the concentration response curves, EC10, and EC50, for seedling

growth for each species tested with a minimum of testing beyond the range-finding test. It is probable that each of the species tested may have a different response curve for a given chemical.

The recommended experimental design is the randomized complete block. As discussed by Hammer and Urquhart (1979), it is essential that the investigator randomly assign plant containers to treatments to assure that each plant has the same chance of receiving any of the treatments (exposure level of test chemical). To account for variation within the chamber and to increase the sensitivity for detecting treatment differences, small square blocks should be delineated in the growth chamber with randomization of treatments within blocks. Replication should occur over chambers (of the same type) as, in many cases, a within-chamber estimate of residual variance badly underestimates the between chamber estimate (Hammer and Urquhart 1979). This means that differences between chambers are often greater than growth and environmental conditions within chambers. In the event that blocking within chambers is impossible, total randomization is acceptable.

In order to substantiate that abiotic test conditions were maintained within specified limits, it will be necessary to measure and record irradiation, photoperiod, temperature, and humidity throughout the test. Requisite instruments are readily available; easily maintained, and should not increase complexity or costs of the test. In fact, these measurements should be made in order to maintain the specified growing conditions.

To standardize procedures and provide guidelines for acceptable data, McFarlane (1979) suggested that radiation measurements be taken at the top of the plant canopy and that a mean, plus a maximum and a minimum value, be obtained over the plant-growing area. These measurements should be taken daily and should be taken and recorded at least at the start and finish of the test. If greenhouse facilities are used, hourly measurements of irradiation should be recorded and presented as daily total irradiance. Representative hourly curves of irradiation for clear sky conditions and cloudy days should also be presented to more fully describe the light climate.

The photoperiod to which each test organism is exposed should be reported. If deviations from the recommended photoperiod occur, they should be recorded so that the potential impact on plant growth can be assessed.

Day/night temperature measurements should be taken at least daily at the top of the plant canopy. Data reported should include the average values for each light and dark period in the study and the range of variation over the growing area.

Humidity should be measured at the same time and location as temperature, and correspondingly reported.

If plants are grown in chambers, it will be necessary to monitor carbon dioxide concentration in the chamber air to ensure that excessive depletions do not occur as a result of rapid photosynthesis rates of the seedlings. These data should also be reported.

Measurement of solution pH prior to testing is recommended to determine if it lies outside the species'

optimal range and contributes to observed effects.

4. Analytical Measurements

The actual chemical concentration used in the definitive test should be determined with the best available analytical precision. Analysis of stock solutions and test solutions just prior to use will minimize problems with storage (e.g., formation of degradation products, adsortion, and transformation). Nominal concentrations are not adequate for the purposes of the definitive tests. If definitive testing is not required because the chemical elicits an insufficient response at the 1000 mg/l level in the range-finding test, the concentration of chemical in the test solution should be determined to confirm the actual exposure level.

The pH of the test solution should be measured prior to testing to determine if it lies outside of the species' optimal range. While it is recognized that plants grow over a broad range of hydrogen-ion concentration and typically exhibit a pH optima for growth, this test guideline does not include pH adjustment for the following reasons: the use of acid or base may chemically alter the test chemical making it more or less toxic, the amount of acid or base needed to adjust the pH may vary from one test solution concentration to the next, and the effect the test substance has on pH may indirectly affect the growth and development of the test plant. Therefore, the pH of each test solution should be determined and compared with the acceptable range for growth and development of the test species.

The data obtained in bioassays are usually expressed as response curves in which growth response of the test species

is plotted against the concentration of the test chemical. The manner of expressing plant response varies considerably. For this guideline, plant growth responses are expressed as direct measurements of length or mass of shoots and roots. The statistical analysis (goodness-of-fit determination) facilitates accurate determination of the EC50 as well as providing confidence limits for the concentration-response curve.

Growth includes both increase in mass (dry weight) and increase in cell number or size (dimensions). Within a test, mass and length are frequently closely correlated and analysis of either produces similar results (Horowitz 1976). Some chemicals, however, induce deformations in either root or shoot systems. Consequently, it is necessary to measure length and mass of roots and shoots in order to adequately assess chemical affects.

C. Test Conditions

1. Test Species

a. Selection

The ten terrestrial plant species recommended for the seed germination/root elongation, early seedling growth, and plant uptake test guidelines are as follows:

Lycopersicon esculentum (tomato)
Cucumis sativus (cucumber)
Lactuca sativa (lettuce)
Glycine max (soybean)
Brassica oleracea (cabbage)
Avena sativa (oat)
Lolium perenne (perennial ryegrass)
Allium cepa (common onion)
Daucus carota (carrot)
Zea mays (corn)

In addition, other species of economic or ecological importance to the region of impact may also be tested in lieu of these species.

These ten species have been selected for the following reasons:

- As food, forage, or ornamentals they are economically important and constitute major cash crops.
- Their distribution, abundance and taxonomic representation suggest broad coverage of the plant kingdom.
- They are also specified for phytotoxicity testing of pesticides (Subpart J, Pesticide Registration Guidelines). Additional justification for selection of these test species is provided in these guidelines (see FR 45(214): 72948-72978).
- They are sensitive to many toxic compounds and have been used to some degree in previous bioassays. Their use in herbicide bioassays, heavy metal screening, salinity and mineral stress tests and allelopathic studies indicates a sensitivity to a wide variety of stressors (Guenzi and McCalla 1966, Geronimo et al. 1973, Puerner and Seigel 1972, Wiedman and Appleby 1972, Reynolds 1978, Chang and Foy 1971).
- They are compatible with the environmental growth conditions and time constraints of the test method. Seed from the selected species germinate quickly and easily. Root growth is rapid and uniform. The seed contain no natural inhibitors

and require no special pretreatment to germinate (such as soaking, chilling, prewashing, light, or scarification).

Other species may be substituted for any or all of these ten species when appropriate. For example, forest or desert species may constitute the population at greatest risk. If so, those of most value to man or of ecological dominance to the affected ecosystem should be selected. The rationale for selection of alternative species should be discussed with the Agency and/or supported in the report of findings.

No single plant will always be the most or least sensitive to all chemicals which may be tested. The use of different types of plants ensures that variations in plant responses will be evident. In a seedling growth test, Hikino (1978) used concentrations of 0.01 to 1,000 ppm for eight chemicals. Rice, turnip and soybean seed were placed in petri dishes with test chemical and agar medium and incubated in the dark. Root and shoot development were measured each day and at the end of the test period. Six of the eight chemicals inhibited root growth at 100 or 1,000 ppm for each species. The other two chemicals inhibited root growth in at least one of the species. It is important to note that none of the three species tested was consistently the "most sensitive". These results further support the requirements of testing several species.

The definitive test requires that seed of the same size class be used to reduce variability in the size of seedlings. Generally, the larger or heavier the seed, the greater the percent germination and the more vigorous the seedling (Pollock and Roos 1972, Smith et al. 1973, Sharples

1970, Whalley et al. 1966, Carleton and Cooper 1972). The differential response to chemical stress response mandates uniformity of seed and seedlings in order to minimize variability. It is recommended that seed be selected from the size class containing the most seed thus providing assurance that sufficient seed are available, that the resulting seedlings will be robust and of uniform size, and that seedling sensitivity to the test chemical is representative of the species.

Information provided by the seed supplier provides additional assurance that the seed are viable. Use of seed produced during one growing season minimizes problems associated with differential viability between lots. By using untreated seed, possibilities of confounding test results with fungicides, repellants, etc. are eliminated.

2. Facilities

a. Apparatus

Greenhouse space, growth rooms, or environmental chambers are equally acceptable as long as the specified environ-mental conditions are maintained. The environment affects growth, metabolism, evapotranspiration, and photosynthesis of plants. Other facilities typically needed for conducting seedling growth tests include standard laboratory glassware, a source of distilled water, work areas to clean and prepare equipment and to measure chemical concentrations and plant responses, drying ovens, refrigeration to hold the seed until needed for testing, and proper disposal facilities. Without these facilities, the testing cannot be adequately conducted.

b. Containers and Support Media

Containers for hydroponic solution or root support media should be composed of inert materials, such as polyethylene, in order to prevent reaction of test chemicals and/or carriers with the container. The root support material should also be composed of inert material for these same reasons. Quartz sand and glass beads are suitably inert materials and do not readily adsorb substances. This lack of adsorption is important because the chemical should be available for root uptake; it would not be as readily available if it were adsorbed to the root support material or container wall.

Sand or glass beads are used, rather than soil, to avoid complications associated with variable physical and chemical properties and microbial populations indigenous to native soils. Native soils are undesirable because of the varying clay, sand, and humus components, the types and proportions of which very within the same soil type. Microbial populations also vary between soil types. These variables alter moisture holding capacity, chemical binding capacity, aeration, and nutrient and trace element content (Audus 1964, Beetsman et al. 1969, Beall and Nash 1969). In addition to the variations in these physical factors, there will also be variation in such chemical properties as pH and redox potential. Because of the impossibility of controlling physical and chemical properties of native soils, inert material is required to support the plants with the only variables being the presence and concentration of test chemical. The purpose of using glass beads or sand instead of native soils is not to make test results more

directly applicable to natural systems, rather it is to eliminate sources of variation in the test.

c. Cleaning and Sterilization

Standard good laboratory practices are recommended to remove dust, dirt, other debris, and organic and inorganic residues from the test containers and support media which might confound test results. Residues could enter the hydroponic or nutrient solution and be taken up by plants, affecting their growth and/or other metabolic activity, resulting in misleading data. A dichromate solution should not be used for washing containers or beads because dichromate may enter the nutrient solution, be taken up by plantss, and affect their growth and metabolic activity.

d. Nutrient Media

The nutrient medium specified in the protocol is modified half-strength Hoaglund solution (Downs and Helmers 1975). This nutrient solution is recommended because all the constituent compounds are relatively easy to obtain and because it works well for the culture of terrestrial plants.

Hydroponic or nutrient culture techniques eliminate spatial gradients within the growth medium and maintain the root system at uniform levels of aeration, nutrients, and water status (Rawlins 1979). The simplest and most practical method for routine plant growth in controlled environments is a sand or gravel culture in which thee plants are grown in containers of sand which are periodically filled to provide water and nutrients, and drained, to provide aeration. The frequency of irrigation depends on the storage capacity of the sand and the rate of water use by the plant. Use of aerated hydroponic solution

without a root support media may be preferred by some laboratories.

Reliability is the primary consideration in the design of the system which automatically fills and drains the containers. One of the simplest systems includes a standpipe connected to an outlet at the bottom of each container through a manifold. The tops of the containers are at a uniform elevation. Periodically, the solution level in the standpipe is raised to the corresponding level and then lowered below the bottom of the containers. This system requires only one conduit to each container (for both filling and draining) and permits variation in the number of containers. The standpipe is filled by a pump from a lower storage reservoir. Electrical power to start the pump and close the drain valve is controlled by a clock timer and, when the nutrient solution reaches the desired level in the standpipe, a float switch turns off the power to the pump and opens the drain allowing the solution to drain back to the reservoir.

If the rate of uptake for test chemicals applied in the nutrient solution differs from the rate of nutrient and water uptake, the exposure concentration will change with time and differ from that originally specified. In addition, chemicals applied to foliage as sprays or dust will enter the rooting zone and accumulate in the nutrient solution. While foliar application is designed to allow for chemical movement through, and uptake from, the rooting zone, excessive concentrations of test chemical should not be allowed to accumulate in the nutrient solution. The test solution should be replaced when or if the test chemical

concentration differs from that specified by ± 20%. When the chemical is applied to the foliage, the nutrient solution should be replaced weekly if detectable quantities move through and accumulate in the solution following spraying or dusting of foliage.

3. Environmental Conditions

Controlled environmental conditions are necessary to maintain uniform growth and ensure reliable data. Maintenance of specified environmental conditions before, during, and after plant exposure to the chemical is essential to the successful execution of this test. Variability of plant response to chemical exposure as a result of fluctuations in environmental conditions has been noted (Darwent and Behrens 1972, Dunning and Heck 1973, Juhren et al. 1957, Leone and Brenann 1970).

Environmental conditions affect growth, metabolism, evapotranspiration, and photosynthesis of plants. In addition to mineral nutrition the conditions that should be standardized and maintained include: (a) carbon dioxide concentration; (b) relative humidity; (c) irradiation; (d) photoperiod; (e) day and night temperatures.

Standardization of environmental conditions is essential. Several investigators have demonstrated that differences in environmental conditions influence the response of plants exposed to chemicals. These include preconditioning by light and humidity (Dunning and Heck 1973), effects of temperature, photoperiod, and light intensity during the growth (Juhren et al. 1957), air movement during exposure (Brenann and Leone 1968), and mineral nutrition (Leone and Brenann 1970). Environmental conditions between

growth chambers should be closely maintained, as specified in this protocol, to ensure common test conditions. If large growth rooms or greenhouse facilities are used, comparability of the environment between small groups of plants is not as critical, and environmental specifications may be relaxed.

a. Carbon Dioxide Concentration

The carbon dioxide concentration should be high enough for photosynthesis to occur at a level which will allow normal plant growth and biomass accumulation. Photosynthetic rates vary directly with carbon dioxide partial pressures. In addition, abnormally high levels of carbon dioxide can affect carbohydrate translocation from the leaves. Therefore, the concentration of carbon dioxide in the air surrounding test plants should be kept within limits conducive to normal plant growth (350 $\pm$ 50 ppm). The carbon dioxide concentration is not expected to be of concern in greenhouse facilities.

In growth chambers, however, carbon dioxide depletion by rapidly photosynthesizing plants may be a real concern. The extent of depletion is a function of chamber volume, rate of air exchange between the chambers and the outside, the number, size, and type of plants, and the growth conditions maintained in the chambers (Hellmers and Giles 1979, Pallas 1979). Attempts to reduce carbon dioxide depletion by increasing the air exchange rate between the outside air and the chamber would probably eliminate temperature and humidity control within the chambers (Downs and Hellmers 1975). An appropriate system for carbon dioxide control in up to 5 chambers is described by Hellmers and Giles

(1979). Alternatively, the use of fewer plants per chamber may be an appropriate means of avoiding carbon dioxide depletion problems.

b. Irradiation

The growth chamber irradiation spectrum should be as similar to natural sunlight as possible in order for test plants to respond normally. Light drives the photosynthetic process and specific wavelengths cause particular responses (e.g., the phytochrome reactions) in plants. Therefore, it is important that light quality and intensity remain constant throughout the test. Irradiation in chambers should be 350 $\pm$ 50 uE/m^2 sec at 400-700 nm measured at the top of the plant canopy. This corresponds roughly to full, direct sun plus diffuse radiation. Artificial lighting is used in growth chambers and as supplemental lighting in greenhouses. However, lamps may weaken, cease to function, or function abnormally and fail in the course of long-term tests. Lamp failures should be corrected as soon as observed and the changes in light quality and intensity resulting from the lamp failures should be recorded.

For light quality and intensity to approach that of natural sunlight, the light source commonly consists of either a fluorescent-incandescent system, a high intensity discharge system (HID) composed of metal halide lamps, or a combination of metal halide and high-pressure sodium lamps. Combinations of lamps are necessary for artificial lighting bcause there is no single lamp which is capable of emanating light of the same quality and intensity as natural sunlight, although the metal halide lamp closely approaches ideal lighting conditions.

HID systems produce intense light that may not be obtainable in other ways (McFarlane 1978). Because these lamps emit radiation in discrete line spectra, researchers frequently combine two different types of HID lamps in growth chambers to obtain a more balanced spectrum. Metal halide lamps appear to be the most useful type of HID lamp because their emission spectra are almost continuous over the 400 to 700 nm wavebands. However, a combination of metal halide and sodium HID lamps will provide superior plant growth to either one alone.

A fluorescent-incandescent system should be composed of 70-80 percent input wattage of cool-white fluorescent lamps and 20-30 percent input wattage of incandescent lamps (Downs 1975). Although incandescent lamps are capable of emanating light which qualitatively approaches natural sunlight, the intensity will not approach the 350+50 uE/m^2 sec specified for plant growth. Increasing the number of incandescent lamps would result in overheating the growth chamber. Fluorescent lighting alone can approach the intensity of natural sunlight without overheating the growth chamber, since very little of the output is in the infrared range (McFarlane 1978). Incandescent lights should be included to provide radiation in the red and infrared regions. Uniformity and intensity of lighting within chambers is routinely improved by covering the walls with highly reflective materials.

c. Photoperiod

Plants exhibit three basic photoperiodic responses. Long-day plants will flower only when the light period is longer than a certain minimum number of hours in a 24 hour

day/night cycle. Short-day plants will flower only when the light period is shorter than a certain maximum number of hours in a 24 hour day/night cycle. Day-neutral plants will flower regardless of the length of the light period. In this test, long day conditions (16 hours light/8 hours darkness) are recommended in order to maximize biomass. Continuous light, however, is not recommended as a dark period is required for the phytochrome interconversions necessary in photosynthetic vascular plants (Salisbury and Ross 1969).

d. Day/Night Temperature

For any particular plant species, there is an optimal temperature regime for maximum growth and development. This regime may differ between the phenophases of germination, vegetative growth, flowering, and fruit development. The concept of thermoperiodicity specifies that the temperature during the light period should be different from that of the dark period for optimal plant growth and development (Downs 1975). It has been demon-strated that plants grow and develop better with a day/night temperature differential (Kramer 1957, Went 1957). While specific temperature optima can be identified for each species, a regime of 25°/20° $\pm$ 3°C is recommended as it will promote suitable growth and be cost effective. If growth chambers or rooms are used, tolerances of $\pm$ 1°C are recommended to ensure environmental comparability between growth facilities.

e. Relative Humidity

A literature review by Hoffman (1979) indicates that most plants grow well when the atmospheric saturation deficit is maintained between 5 and 10 mb. At 25°C, the

optimum relative humidity range is 65 to 85 percent. Most environmental control facilities with provision for humidity control can maintain humidity in this range with an accuracy at 25°C of ± 1.0 to 1.6 mb of vapor pressure (± 3 to 5 percent relative humidity). Humidity levels have been observed to affect plant growth and development, rates of carbon dioxide exchange, flowering, nutrient transport, and susceptibility to air pollution (Thurtell 1979). However, these are generally the responses of plants to their total environments and are not unique to the humidity of the air. During the daylight period the relative humidity in the growth chamber should be maintained within the optimal range of 65 to 85 percent. When several chambers should be maintained as comparable environments, a relative humidity of 70 ± 5 percent is recommended. During the dark period, relative humidity should approach saturation, as it normally does in natural environments.

D. Reporting

The sponsor should submit to the Agency all data developed during the test which are suggestive or predictive of phytotoxicity. If testing specifications are followed, the sponsor should report that specified procedures were followed and present the results. If alternative procedures were used instead of those recommended in the test guideline, then the protocol used should be fully described and justified.

Environmental test (growth) conditions, chemical concentrations, quantity of chemical applied, number of applications, test data, concentration response curves, and statistical analyses should all be reported. The

justification for this body of information is contained in this support document. If plant species other than the ten recommended were used, the rationale for the selection of other species should also be provided.

III. Economic Aspects

The Agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for performing an early seedling growth test according to this guideline. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

The protocol estimate was $1,184. This estimate was prepared by identifying the major tasks needed to do a test and estimating the hours to accomplish each task. Appropriate hourly rates were then applied to yield a total direct labor charge. An estimated average overhead rate of 115%, other direct costs of $338, a general and administrative rate of 10%, and a fee of 20% were then added to the direct labor charge to yield the final estimate.

Enviro Control estimated that differences in salaries, equipment, overhead costs and other factors between laboratories could result in as much as 50% variation from this estimate. Consequently, they estimated that test costs could range from $592 to $1,776.

The laboratory survey estimate was $12,938, the mean of the estimates received from two laboratories. The estimates ranged from $9,600 to $16,000 and were based on the costs to perform the test according to this Guideline.

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EG-14
August, 1982

PLANT UPTAKE AND TRANSLOCATION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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PLANT UPTAKE AND TRANSLOCATION TEST

(a) Purpose. The guideline in this section is intended for use in developing data on the uptake and translocation of chemical substances and mixtures ("chemicals") by terrestrial plants subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003, 14 U.S.C. 2601 et. seq.). This guideline prescribes tests using commercially important terrestrial plants to develop data on the quantity of chemical substances incorporated in plant tissues and the potential for entry into food chains with resultant indirect human exposure. The United States Environmental Protection Agency (USEPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA), and Part 792--Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this guideline:

(1) "EC X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Mass balance" means a quantitative accounting of the distributions of chemical in plant components, support medium, and test solutions. It also means a quantitative determination of uptake as the difference between the quantity of gas entering an exposure chamber, the quantity leaving the chamber, and the quantity adsorbed to the chamber walls.

(3) "Support media" means the sand or glass beads used to support the plant.

(4) "Translocation" means the transference or transport of chemical from the site of uptake to other plant components.

(c) Test procedures--(1) Summary of the test--(i) Root exposure. In preparation for the test, seeds are planted in the potting containers (or in cotton or glass-wool plugs supported in hydroponic solution) and, after germination, seedlings thinned, by pinching the stem at the support surface. Potting mixtures of sand or glass beads should be sub-irrigated with nutrient solution. Chemicals are applied to the plants via nutrient solution or adsorbed to the support media. Carrot, lettuce, onion, cabbage, and ryegrass may be harvested whenever there is adequate plant material for chemical analysis. Cucumber, corn, soybean, tomato, and oat should be grown until fruit or seed are mature.

(ii) Foliar exposure. The foliar exposure test is identical to the root exposure test except that chemicals are applied to plants by either spraying or dusting the foliage or exposing the plants to gas in a fumigation chamber. If plants are fumigated, either rates of uptake and surface adsorption should be calculated, or the plants may be harvested and analyzed for test chemical and residues.

(2) Chemical Application--(i) Root exposure. (A) Chemicals that are soluble in water should be dissolved in the nutrient solution just prior to the beginning of the test. Deionized or glass distilled water should be used in making stock solutions of the test chemical. Sufficient quantities of each concentration should be made up as needed to minimize storage time and disposal volume.

(B) Chemicals that are insoluble in water but which can be placed in aqueous suspension with a carrier should be added, with the carrier, to the nutrient solution. The carrier should be soluble in water, relatively non-toxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol, and others have been used extensively in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants.

Carrier controls should be included in the experimental design and tested simultaneously.

(C) Water-insoluble chemicals for which no non-toxic, water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution should be mixed with the sand or glass beads which are then placed in a rotary vacuum apparatus and evaporated, leaving a uniform coating of chemical on the sand or beads. A weighed portion of beads should be extracted with the same organic solvent and the chemical assayed before the potting containers are filled. Solvent controls should be included in the experimental design and tested simultaneously.

(ii) Foliar exposure. (A) Water soluble chemicals should be dissolved in deionized or glass distilled water just prior to use. Sufficient quantities of each concentration should be made up as needed. These solutions should be applied at weekly intervals. Plants should be placed in an exhaust hood and the chemical applied to the foliage. A plastic sleeve may be fitted over the top of the pot, and the foliage sprayed with specific quantities of test solution at known concentrations. The plastic sleeve, confining the chemical to plant and pot, facilitates expression of chemical dosage as quantity per pot area (i.e., ug/m^2). Shoots of control plants should be sprayed in an

identical manner with deionized or distilled water. Alternatively, a miniature compressed-air sprayer may be mounted on a pendulum and equipped to automatically spray a plant positioned directly beneath the center of its arc of swing. When radioisotope-labelled chemicals are applied, health and safety considerations prohibit spray application. Instead, specific quantities of labelled chemical should be applied directly to leaves in single drops.

(B) Water-insoluble chemicals, existing as solids, may be prepared for testing by grinding or other reduction to particles of <200 um diameter. These chemicals should be applied at weekly intervals. Plants should be placed in an exhaust hood, a plastic sleeve fitted over the top of the pot, and a specific quantity of chemical sprinkled uniformly over them. Prior to chemical application, plants should be misted with water to promote foliar retention of the chemical. Control plants also should be misted with deionized or distilled water at each treatment date and dusted with an inert material of the same particle size. Applications should be expressed as quantity per unit pot area (i.e., ug/m^2).

(C) Chemicals existing in gaseous form at normal ambient temperatures and pressures should be generated for use as needed or stored under pressure. The bottled gas may be 100 percent

pure chemical or mixed with an inert carrier, such as nitrogen, to known concentrations. Chemicals of controlled or measured concentrations should be metered into the exposure chamber, uniformly mixed about the plants, and exhausted through the outlet port where the flow rate and concentration are again measured. Use of this system design provides an alternate method of analysis if the quantity of chemical sorbed by plants is less than that required for chemical analysis. Plants should be fumigated whenever they have reached sufficient size for measurement of photosynthesis and transpiration rates, assuming equivalent detection sensitivity of carbon dioxide, water vapor, and chemical analyzers. The appropriate size is a function of the gas exchange system and constitutes an area of expert judgement.

(3) Range-finding test. (i) A range-finding test should be conducted to establish the chemical concentrations used in the uptake and translocation test.

(ii) Because of the different mechanisms involved in root and leaf uptake, and to more closely define the chemical concentrations to be used in the uptake test, the definitive early seedling growth test is recommended as the range-finding test. Seeds should be germinated directly in containers filled with sand or glass beads or in cotton or glass-wool plugs

supported in hydroponic solution. When 50 percent of the seedlings have germinated, the seedlings should be thinned (by pinching) to the 10 most uniform per container and exposed to a concentration series of test chemical. The lowest concentration in the series, exclusive of controls, should be at or below the EC 10 while the upper concentration should be at or above the EC 90. If the anticipated fate of the chemical is soil or soil-water, and the mechanism of concern is root uptake, the chemical should be applied in nutrient solution to the root support media (or coated on sand or glass beads for non-water soluble chemicals). With a chemical whose anticipated mode of exposure to plants is surface deposition by atmospheric transport or irrigation water, the appropriate testing method may be foliar application allowing subsequent movement into the rooting zone with watering. Effect is assessed as growth reduction. The concentration selected as the upper limit for the uptake and translocation test should be near the threshold of visible injury. Short exposure periods to gas in fumigation chambers are not expected to promote visible injury or gross reductions in growth but may alter stomatal resistance, transpiration, or photosynthesis. Absorption and adsorption rates may be calculated and gas concentrations for definitive testing selected based on the calculated sorption rates.

(iii) Alternatively, the seed germination/root elongation test or other appropriate phytotoxicity test may be used to establish the appropriate upper concentration for testing.

(4) Definitive test. (i) The purpose of the uptake and translocation test is to determine the propensity for a chemical's accumulation in plants or plant parts.

(ii) At least 3 concentrations of chemical, exclusive of controls, should be used in the uptake test. Recommended concentrations would be a descending geometric progression from the upper concentration tested (i.e. 100, 50, 25 mg/l). A minimum of 6 replicate pots per concentration, each containing from one to four seedlings, should be used. If techniques other than radioisotopes are used to determine uptake, more replicates may be required to provide sufficient plant materials for analysis. Test chemicals should be added to the hydroponic or nutrient solution or coated on glass beads for the root uptake test; or sprayed, dusted, or gassed directly on the foliage in the foliage uptake tests. Only untreated seed (not treated with fungicides, repellants, etc.) taken from the same lot, and year or season of collection should be used in a given test.

(iii) Control pots should be included in the experimental design and should be used in each run. In addition, a carrier control should be used for those chemicals that need to be

solubilized.

(iv) If plants are to be grown hydroponically, seeds should be planted in plugs of cotton or glass-wool supported in the tops of the containers. When sand or glass beads are used, the recommended planting procedure is to fill potting containers with sand or glass beads to within 2.5 cm of the top and to sow seeds directly. After germination, the seedlings should be thinned by pinching the stem at the support surface. From one to four seedlings per potting container are required depending on species tested, the size of the containers, and the size to which the plants will grow. When plants are grown hydroponically, one plant per pot will probably be the preferred method. The number of plants selected should provide sufficient biomass for analytical procedures. A greater number of plants may be required depending on species tested, duration of test, and analytical procedures. Too many plants in a container may actually reduce the growth and biomass.

(v) Alternate planting methods may be required when the chemical is highly volatile. An impervious barrier of polyethylene film, a modification of the double pot method, a glass plate, or other appropriate apparatus should be used to prevent volatilization from the root zone. Seeds should be germinated in the dark at 25°C and seedlings with radicle length

in the median range transplanted into the potting containers. The seedlings should be positioned such that their roots are exposed to the support media while the shoots pass through holes in the barrier. A ring of inert, non-phytotoxic, pliable putty should be used to seal the holes around the stems. Control pots should be handled identically except there is no exposure to the test chemical. This transplanting procedure, without the volatilization barrier, is also recommended when the test chemical is adsorbed to the support medium.

(vi) Hydroponic solutions should be aerated and sand or glass filled potting containers should be periodically filled with nutrient solution and drained to provide aeration. For root exposure tests, the test chemical should be added to the nutrient solution or directly to substrate. The entire test solution should be replaced weekly, or earlier if the concentration of chemical in the test or nutrient solution varies by more than 20 percent of that specified. The volume of solution added should be recorded.

(vii) The test consists of one run for each of two specified plant species. The duration of a run, for solid and liquid chemicals, should be equal to the length of time required for the particular test variety to achieve sufficient biomass for testing. The duration of a run for gasseous chemicals

should the length of time required to make the specified gas exchange measurements. For a particular chemical, a run is defined as exposure of the plant species to three concentrations of test chemical with a minimum of 6 replicate pots and appropriate controls. Exposure is followed by extraction and analysis for parent compound, metabolites, and bound residues in plant tissues, and in the whole plants for solids, liquids, and gasses or by calculating rates of absorption and adsorption of gasses.

(viii) Visible effects (stunting of growth, discoloration, chlorosis and/or necrosis of the leaves, decreased moisture content, or morphological abnormalities, etc.) should be recorded.

(ix) A randomized complete block design is recommended for this test, with blocks delineated within the chambers or over greenhouse benches and randomization of treatments occurring within the blocks. If, because of very large pots and plants, there exists inadequate space within chambers for blocking, total randomization within chambers is acceptable. This design is also appropriate for the growth of plants to be used for foliar exposure with gas.

(x) Irradiation measurements should be taken at the top of the plant canopy and the mean, plus a maximum and a minimum value, determined over the plant-growing area. These

measurements should be taken at the start of the test, at biweekly intervals during the test, and at test termination. If the test is conducted in a greenhouse facility, hourly measurements of irradiation should be recorded and presented as daily total irradiance plus representative hourly curves for clear sky conditions and cloudy days.

(xi) Temperature and humidity measurements should be measured daily at the top of the plant canopy during each light and dark period.

(xii) Measurements of carbon dioxide concentration should be made at the top of the plant canopy (of chamber-growth plants) on a "continuous" basis.

(xiii) The amount of water and nutrient solution depleted each week should be recorded, to observe changes in evapotranspiration rates which may indicate stress. Furthermore, these data will be used to compute chemical uptake per volume of water transpired for the uptake test.

(5) [Reserved]

(6) Analytical measurements--(i) Solid or liquid test chemicals. (A) Stock solutions should be diluted with glass-distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be

validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(B) The entire plant should be harvested, rinsed with a minimum amount of water (which is returned to the nutrient solution), and separated into its respective organs as follows: carrot - root peels, peeled roots, and tops; cucumber - fruit, vines plus leaves, and roots; corn - kernels, husk plus cob, stalk plus leaves, and roots; lettuce - tops and roots; onion - bulb and tops; ryegrass - tops and roots; soybean - grain, chaff plus tops, and roots; oats - grain, chaff plus tops, and roots; tomato - fruit, vines, and roots; cabbage - head and roots. Plants from two pots in each treatment may be pooled, giving 3 replicate sample pools per treatment. After the fresh weights of the plant organs are obtained, each pool of organs should be subsampled for percent moisture determinations by drying, at 70°C for 24 hours in a forced-air drying oven, and weighing. Percent moisture determined from these subsamples is used to correct for dry weight of the fresh samples which should then be homogenized and extracted in organic and aqueous solvents. If radioisotopes are used, the amount of test chemical in each extract should be

determined by liquid or solid scintillation depending on the type of radiation; otherwise, the amount of chemical should be determined by standard methods. At test completion, the root support material should be washed in organic and then aqueous solvent and analyzed for test chemical before discarding.

(C) A suggested extraction procedure appropriate for many organic chemicals is as follows: One gram of plant material should be homogenized with one gram of solvent-washed anhydrous sodium sulfate in 4 ml of hexane or acetonitrile. The homogenate should be filtered or centrifuged, the solid residue rinsed with an appropriate organic solvent, and the filtrate or supernatant combined with the rinse. The solid residue should be extracted by sequentially (1) homogenizing in water, (2) centrifuging and decanting the supernatant, (3) extracting of the pellet with 6N hydrochloric acid at 60°C for 10 hours, (4) subsequently digesting with 10N potassium hydroxide, and (5) combining supernatants. The resulting solution should be analyzed by liquid scintillation spectrometry or GLC methodology. The organic extract should be evaporated under vacuum to a sufficiently small volume for thin layer chromatography (TLC) and co-chromatographed on silica gel plates with known standards of the parent chemical. If radioisotopes were used, the chromatographs could be scanned for radioactive substances on a radiochromatogram scanner. Alternatively, zones may be

removed from the plates, extracted, and the quantity of chemical from each zone determined by liquid scintillation spectrometry or GLC methodology. The unextractable chemical in the remaining residue may be determined by oxidizing the residue in a complete combustion oxidizer.

(ii) Gaseous test chemicals. (A) A gas exposure system yields requisite data for a direct calculation of uptake rates. At steady state, chemical uptake may be determined by a mass balance calculation. Correction for adsorption to surfaces of the exposure chamber should be made by operating the system without plants. Pots filled with hydroponic solution or support media should be included in the system adsorption calibration. Consequently, chemical analyses of plant tissues exposed to gaseous chemicals may not be required in order to demonstrate and quantitate uptake rates.

(B) Altered rates of net photosynthesis, transpiration, and stomatal conductance are anticipated as a result of chemical uptake. Rates of these physiological processes before, during, and after exposure to the gaseous chemical should be determined. Data required for these calculations are available as a consequence of maintaining the specified environmental conditions within the fumigation chamber.

(iii) Numerical. Mass of pooled plant organs and pooled whole plants should be measured for the uptake and translocation test and subjected to chemical analyses (above) to quantify free parent test chemical, its metabolites and soluble and bound residues. Mass balance of the test chemical and evapotranspiration rates of the plants are also determined. Means and standard deviations should be calculated and plotted for each of the above for every treatment and control. The data should also be subjected to an analysis of variance.

(d) Test conditions--(1) Test species. (i) Test plants recommended for the uptake test include:

Lycopersicon esculentum (tomato)

Cucumis sativus (cucumber)

Lactuca sativa (lettuce)

Glycine max (soybean)

Brassica oleracea (cabbage)

Avena sativa (oat)

Lolium perenne (perennial ryegrass)

Allium cepa (common onion)

Daucus carota (carrot)

Zea mays (corn)

(ii) Other species of economic or ecologic importance to the region of impact, may also be appropriate and selected for

testing. Two species of potentially differing sensitivity should be selected such as a monocotyledonous and a dicotyledonous species. It is further suggested that the test plants selected should be of different growth forms, e.g., a root crop and a leaf crop.

(2) Facilities--(i) Apparatus. Greenhouses, environmental chambers, or growth rooms should provide adequate environmental control to meet the carbon dioxide, humidity, irradiation, photoperiod, and temperature specifications. Chambers should be designed to prevent escape of internal air into the external environment other than through appropriate filtering material or media to prevent contamination of the external environment with radioactive and/or test substances. Laboratory facilities for plant extractions and chemical determinations should include non-porous floor covering, absorbant bench covering with non-porous backing, and adequate disposal facilities to accommodate plant nutrient, test, and wash solutions containing radioisotope and/or test chemical at the end of each run, and any bench covering, lab clothing, or other contaminated materials.

(ii) Containers and support media. For testing purposes, at least 24 polyethylene pots sufficiently large to grow at least 5 plants up to 28 days or one to three plants to maturity are required. If plants are grown hydroponically, one plant per pot

may be the preferred method. If a carrier control is needed, 30 pots are used. Potting containers used in each experiment should be of equal size and volume and possess the same configuration. When sand or glass beads are used the potting containers should be filled to within 2.5 cm of their tops with sand or glass beads. Perlite, vermiculite, native soils, etc., should not be used for root support. Potting containers should be covered with opaque polyethylene bags to exclude light and minimize volatilization of test chemical.

(iii) Cleaning and sterilization. Potting containers, nutrient storage containers, and root support medium should be cleaned before use. All equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or use. A dichromate solution should not be used for cleaning beads or pots. Rooting media other than glass beads should be discarded at the end of the experiment. Disposal should conform to existing regulations.

(iv) Nutrient media. Half-strength modified Hoagland nutrient solution should be utilized as nutrient media for this test. Hydroponic solution should be aerated and sand or glass beads potting containers should be filled with nutrient solution and drained periodically. An automated system design is recommended.

(3) Test parameters. Environmental conditions should be maintained as specified below:

(i) Carbon dioxide concentrations at 350 ± 50 ppm.

(ii) Relative humidity approaching 70 ± 5 percent during light periods and 90 percent during dark periods.

(iii) Irradiation, measured at 1 meter from the source, at 350±50 uE/m^2 sec at 400-700 nm.

(iv) Photoperiod of 16 hours light and 8 hours darkness for all species except soybean which should be provided with 11 hours light and 13 hours darkness prior to flowering.

(v) Day/night temperatures at 25°/20° ± 3°C.

(e) Reporting. Reporting requirements of Part 792--Good Laboratory Practice Standards apply to this guideline. Concentrations should be expressed in appropriate weight units per grams of dry plant material and of water lost by evapotranspiration. Data should also include initial and final total concentration of the test chemical in the growth media. These data will be used to compute mass balance. The following should be reported for each of the species tested in tabular form:

(1) Solid and liquid test chemicals. (i) Concentration of chemical in nutrient solution and root support material when chemical is soluble in water or solubilized with a carrier

compound, as well as the concentration of carrier compound in nutrient solution when carrier is used, or the quantity of chemical per unit weight of root support material when it is coated on the material.

(ii) The quantity of chemical, the concentration at which it was applied, and the number of applications for those chemicals applied to the foliage.

(iii) Environmental conditions (day/night temperatures, relative humidity, light intensity, carbon dioxide concentration, and photoperiod) and the occurrence and extent of any disruption of environmental control facilities.

(iv) Mass of each pool of plant organs and by summation, the mass of whole plants (dry weight after 24 hours at 70°C).

(v) Concentration of free parent test chemical, metabolites and soluble residues, and bound residues in pooled plant organs and pooled whole plants.

(vi) Mass balance of chemical.

(vii) Mean evapotranspiration rate per plant.

(viii) Visible effects of chemical, if any, on the intact plants.

(ix) Analysis of variance, F-test, means, and standard deviation about the mean are calculated under paragraph (e) (1) (iv), (v) (vi), and (vii) of this section.

(2) Gaseous test chemicals. (i) Concentration of gaseous test chemical at inflow and outflow ports.

(ii) Environmental conditions within gas exposure system (air temperature, dew point temperature or water vapor pressure of incoming and outgoing air streams, light intensity, air speed within chamber, carbon dioxide concentration at inflow and outflow ports, gas flow rate into and out of exposure system).

(iii) Mass (dry weight after 24 hours at 70°C) of leaves and stems and surface area (one side of leaves) in the exposure system.

(iv) Calculated measurements of photosynthesis, transpiration, and stomatal conductance before, during, and after exposure to test chemical.

(v) Visible effects of chemical, if any, on the plants.

(vi) Analysis of variance, F-test, means, and standard deviation about the mean are calculated for each of the following: (A) Steady state rates of photosynthesis, transpiration, and chemical uptake before, during, and after fumigation.

(B) Stomatal conductance or leaf diffusion resistance before, during, and after fumigation.

(vii) If uptake is determined by direct chemical analysis of

plant tissues, then the reporting requirements also include:

(A) Concentration of free parent test chemical, metabolites and soluble residues, and bound residues in pooled plant organs and pooled whole plants.

(B) Mass balance of the chemical.

(C) Analysis of variance, F-test, means and standard deviation about the mean under paragraph (e)(2)(A) and (B).

ES-11
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

PLANT UPTAKE AND TRANSLOCATION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TECHNICAL SUPPORT DOCUMENT FOR PLANT UPTAKE AND TRANSLOCATION TEST

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-14 which uses various plant species to evaluate the uptake and translocation of chemical substances in plants. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. General

Chemicals of concern may be transported to various sites as gasses or dust, solubilized in precipitation or in irrigation water, or may be encountered in soils. It is well known that plants readily take up, translocate, accumulate, and metabolize chemicals that are nonessential for plant growth and development. Such uptake and incorporation often represents the first step in the transport of these chemicals within terrestrial food webs. The uptake and translocation test addresses these concerns of entry into food webs. This test is not concerned with phytotoxicity; it deals with hazard to animals and the potential for indirect human exposure.

The plant uptake test was selected for the following reasons:

- The incorporation of chemicals in plant tissues, at concentrations not evidencing visible injury to the plant, may provide an unrecognized hazard to humans and other animals.
- The test measures the quantity of chemical adsorbed to and absorbed by and translocated within terrestrial plants.
- The test can be used to evaluate the potential for human exposure to toxic compounds through terrestrial food webs.
- The data developed, in conjunction with toxicity data and information on chemical fate and transport, provide an important segment of the scientific basis for risk assessment.

The test design is a composite of prior plant experiments in which uptake of heavy metals, pesticides, or organic chemicals have been investigated (Beall and Nash 1971, Cole et al. 1976, Chou et al. 1978, Fuhr and Mittelstaedt 1980, Kelly et al. 1980, Wickliff et al. 1980).

B. Test Procedures

1. Chemical Application

Chemicals have different physical and chemical properties which will influence the mode of application. Water-soluble chemicals will not present a problem but other chemicals will require different approaches. In this section we have attempted to provide general guidance by suggesting some approaches that have been used successfully in the past. There is a need for expert scientific judgment

in the choice of solvent. The Agency recognizes the need to maintain flexibility, yet ensure that any effects are due to the test chemical and not the carrier. The suggested use of carrier controls to distinguish between test chemical and carrier effects is considered a standard laboratory practice.

Water-soluble chemicals are easiest to apply since roots may absorb chemicals directly from hydroponic or nutrient solutions and since many air-borne chemicals arrive in precipitation. Water-insoluble chemicals require other procedures to insure contact between the test chemical and the root and shoot systems. Appropriate controls are necessary and are designed to reduce as much as possible, the number of variables in the system. One means of incorporating a water-insoluble chemical into the hydroponic solution or the nutrient medium is to solubilize it in the solution with a surfactant or carrier compound. When a carrier is used, a carrier control is included in the procedure to check the carrier's effects on plant growth. A minimal quantity of carrier should be used to minimize carrier effects.

Foliar applications of powders or sprays should be done under a hood to ensure worker safety and to minimize growth chamber contamination. Control plants should also be moved from chambers to the hood area and sprayed with distilled, deionized water whenever the chemical is applied to other plants by spraying because plants are highly sensitive to mechanical stresses such as handling (Wheeler and Salisbury 1979).

Attention to maintenance of environmental conditions during applications of gasses is essential to success of the test. Brenann and Leone (1968) observed that plants exposed to identical concentrations of pollutants at different gas flow rates exhibited different toxicity symptoms. These observed variations are understandable when considered in terms of the effect of environmental physics on mass transport to the leaves and on the physiological condition of the plants. In order that each plant in the gas exposure chamber receive the same dosage, chamber design should provide a spatially uniform test chemical distribution. Movement of gaseous substances to the leaf surfaces is dependent upon air turbulance around the leaves reducing the boundary layer thickness (Fowler and Unsworth 1974, O'Dell et al. 1977). Some substances may impact or condense on the surface of the plants (Fowler and Unsworth 1974) while others may diffuse through stomatal pores or across the cuticle to the leaf's interior (Black and Unsworth 1979, Rich et al. 1970). The cuticle provides an effective barrier for gaseous substances and several investigators have shown chemical absorption to be regulated by the same factors that control stomatal opening including light, temperature, humidity, carbon dioxide concentration, and plant water status (Bennett et al. 1973, Black and Unsworth 1979, Rich et al. 1970, Winner and Mooney 1980). Specified environmental conditions allow for normal stomatal response and plant growth. These conditions should be maintained during plant exposure to chemicals. The exposure chamber designed by Rogers et al. (1977) meets the environmental criteria and the criteria of uniform gas distribution within the chamber.

The growth medium control provides a baseline check of the effects of hydroponic solution, potting material, nutrient solution, and the testing environment on plant growth during each test. This control is simply the plant growing in the hydroponic solution or root support material and maintained by the nutrient solution without test chemical, solvent, or carrier. This control is required in each run regardless of whether or not the chemical is soluble in water. It is the only control used when the chemical is water-soluble and is applied directly to the support media or is applied as foliar dust or spray.

A second control is also recommended whenever a carrier (solubilizer, or non-aqueous solvent) is used to maximize contact between the chemical and the plant. The solvent or carrier control is used to check carrier effects on plant physiology and growth and on processes affecting chemical uptake and translocation.

If chemical volatilization is anticipated, a modification of the double pot method is used in which germinated seedlings of uniform size are transplanted into the potting containers; an impervious barrier of polyethylene film is placed over the root support material and seedlings are positioned such that their roots are exposed to the support material and are sealed in place with a ring of non-toxic, inert, pliable putty.

2. Range-Finding Test

It is recommended that a range-finding test be conducted prior to the uptake and translocation test to reduce the risk of using an inappropriate concentration series in the definitive test. In order to minimize the costs and time

required to obtain the requisite data, nominal concentrations are permitted, replicates are not required, and other test procedures and conditions are relaxed.

It may be possible to use results of previously conducted plant effects tests rather than conducting a range-finding test. For instance, results of the seed germination/root elongation test, the early seedling growth test, or other appropriate phytotoxicity tests may be used to establish the appropriate upper concentration for testing.

3. Definitive Test

The specific requirements of the uptake and translocation test are the analytical determinations of chemical con-centrations, the selection of uniformly sized seedlings, the unbiased selection of seedlings for each treatment, the use of controls, and the recording, analysis, and presentation of data. These requirements assure that the propensity for a chemical to accumulate in plants or plant parts is accurately elucidated, that uptake and translocation phenomena are not confounded by differential plant vigor, and that the results are clearly presented. Reporting the occurrence of such abnormalities as stunting, discoloration, chlorosis and/or necrosis of leaves, etc. provides qualitative data that may help explain deviations from expected uptake trends.

The purpose of the uptake and translocation test is to determine the propensity of a chemical to accumulate in plants or plant parts for each species tested with a minimum of testing beyond the range-finding test. The concentration range for the uptake and translocation test is based upon

the results of the range-finding test for that species. It is probable that each species tested will require a different exposure concentration series and will exhibit different uptake and translocation rates.

Plants from 2 of the 6 pots per treatments are pooled, giving 3 replicate sample pools per treatment. The purpose of pooling the plants is to insure that there is sufficient sample biomass for all the specified analyses. Since the reported results should include a statistical summary of the data, plants from all 6 pots per treatment cannot be pooled into one sample pool. However, if chemical recovery is insufficient to permit measurement (not due to sensitivity of the analytical method), a single analysis of pooled material from all six pots may be required to confirm lack of uptake.

The randomized complete block design is the recommended experimental design for the uptake and translocation test. If growth chambers are used, it is essential that the investigator randomly assign plant containers to treatments to assure that each plant has the same chance of receiving any of the treatments (exposure level of test chemical) (Hammer and Urquhart 1979). To account for variation within the chamber and to increase the sensitivity for detecting treatment differences, small square blocks should be delineated with randomization of treatments within blocks. Replication should occur over chambers (of the same type) as, in many cases, a within-chamber estimate of residual variance badly underestimates the between chamber estimate (Hammer and Urquhart 1979). This means that differences between chambers are often greater than differences in

growth and environmental conditions within chambers. In the event that blocking within chambers is impossible, total randomization is acceptable.

In order to substantiate that abiotic test conditions were maintained within specified limits, it will be necessary to measure and record irradiation, photoperiod, temperature, and humidity throughout the test. Requisite instruments are readily available, easily maintained, and should not increase complexity or costs of the test. In fact, these measurements should be made in order to maintain the specified growing conditions.

To standardize procedures and provide acceptable data, McFarlane (1979) suggested that radiation measurements be taken at the top of the plant canopy and that a mean, plus a maximum and a minimum value, be obtained over the plant-growing area. These measurements should be taken daily and should be taken and recorded at least at the start and finish of the test. If greenhouse facilities are used, hourly measurements of irradiation should be recorded and presented as daily total irradiance. Representative hourly curves of irradiation for clear sky conditions and cloudy days should also be presented to more fully describe the light climate.

The photoperiod to which each test organism is exposed should be reported. If deviations from the recommended photoperiod occur, they should be recorded so that the potential impact on plant growth can be assessed.

Day/night temperature measurements should be taken at least daily at the top of the plant canopy. Data reported should include the average values for each light and dark

period in the study and the range of variation over the growing area.

Humidity should be measured at the same time and location as temperature, and correspondingly reported.

If plants are grown in chambers, it will be necessary to monitor carbon dioxide concentration in the chamber air to ensure that excessive depletions do not occur as a result of rapid photosynthesis rates of the seedlings. These data should also be reported.

4. Analytical Measurements

It is imperative that the actual concentration of chemical to which plants are exposed be determined with the best available analytical precision. Analysis of stock solutions and test solutions just prior to use will minimize problems with storage such as formation of degradation products, adsorption to container walls, or chemical transformations. Nominal concentrations are not adequate for the purposes of the definitive tests.

The pH of the test solution should be measured prior to testing to determine if it lies outside of the species' optimal range. While it is recognized that plants grow over a broad range of hydrogen-ion concentration and typically exhibit a pH optima for growth, this test guideline does not include pH adjustment for the following reasons: the use of acid or base may chemically alter the test chemical making it more or less toxic, the amount of acid or base needed to adjust the pH may vary from one test solution concentration to the text, and the effect the test substance has on pH may indirectly affect the growth, development, and uptake processes of the test plant. Therefore, the pH of each test

solution should be determined and compared with the acceptable range for growth and development of the test species.

Harvested plants are rinsed with water to remove any surface depositions of the test chemical. This rinse water is retained for purposes of the mass balance calculations. The plants are separated into component parts representative of use. Plant component parts are pooled to provide sufficient material for chemical analysis. Subsamples of the pooled plants are oven dried and weighed; and dry weight equivalents are calculated for each component. This procedure avoids the potential volatilization of organic chemicals from the plant tissues during the drying process.

Because the chemical may be differentially soluble in organic and aqueous solvents, both are included in the extraction procedure. The nature of the organic solvent used will depend upon the particular chemical properties. Hexane or acetonitrite is suggested in the homogenization procedure. They are commonly used organic solvents and most organic compounds will dissolve in them. Anhydrous sodium sulfate is used to absorb water from the mixture and minimize moisture complications in the procedure. The organic solution is separated from the homogenate and may be analyzed by liquid scintillation spectrometry in order to determine the amount of radioactive compound in the extractable organic portion.

If the chemical is present in the organic extract, it can be identified and quantified by thin-layer chromatography followed by autoradiography of the thin-layer plates and/or counting with a radiochromatogram scanner.

A water soluble chemical would be expected to appear primarily in the aqueous extract of the plant sample homogenate. Therefore, chemical analysis of the aqueous extract is also conducted as above.

Metabolites and soluble residues of the chemical may also be present in the organic and aqueous extracts of plant homogenates. Liquid scintillation counting of the extracts followed by evaporation and thin-layer chromatography provides a measurement of metabolites and soluble residues of appropriately labeled chemicals but does not allow for differentiation between chemical forms.

Bound residues are those forms of the chemical in the plants that are not extractable in either organic or aqueous solvents. Plant tissues should be digested in some manner before bound residues can be measured. Three methods are available: (1) digestion with a tissue solubilizer, (2) complete combustion oxidation and collection of carbon dioxide and (3) wet digestion. When the test chemical is labeled with ^{3}H or ^{14}C, either digestion with a tissue solubilizer or complete combustion oxidation is applicable. Of the two, combustion oxidation is preferred, because tissue solubilization does not dissolve all material at the same rate and may result in quenching and interference in the scintillation solution. This problem does not appear in the carbon dioxide solution resulting from combustion oxidation. When the test chemical is labeled with 32P or 35S, either digestion with a tissue solubilizer or wet digestion is appropriate. Wet digestion is preferred for 32P or 35S determination because it results in a more complete digestion of plant tissue than occurs with a tissue solubilizer.

Although it may be possible to determine plant uptake of gases by analytical chemistry, a direct mass balance calculation during plant exposure may be preferred. This would completely eliminate the need to do chemical analyses of plant tissues and be very time and cost effective. Measured rates of photosynthesis and transpiration prior to fumigation are essential in order to establish that environmental conditions in the exposure chamber are conducive to normal physiological processes and that the stomates are open. In addition, this establishes a base level rate (zero chemical concentration) of important physiological processes. Rates of photosynthesis during and after fumigation provide indications of chronic phytotoxicity and, with data on stomatal conductivity, also allow considerations of mechanism of chemical action and of plant resistance to the chemical (Winner and Mooney 1980). Details of equipment requirements and calculations for gas exchange/fumigation studies can be found in Bennett et al. (1973), Black and Unsworth (1979), Keller (1980), O'Dell et al. (1977), Rogers et al. (1979), Thompson et al. (1979), and Winner and Mooney (1980).

The mass of pooled plant organs and pooled whole plants, the mass of chemical in each plant pool, rates of transpiration and photosynthetis, and values of stomatal conductivity (when determined) are analyzed. Means and standard deviations are calculated and plotted for each treatment and control and the data are subjected to analysis of variance.

C. Test Conditions

1. Test Species

a. Selection

The ten terrestrial plant species recommended for the seed germination/root elongation, early seedling growth, and plant uptake test guidelines are as follows:

Lycopersicon esculentum (tomato)
Cucumis sativus (cucumber)
Lactuca sativa (lettuce)
Glycine max (soybean)
Brassica oleracea (cabbage)
Avena sativa (oat)
Lolium perenne (perennial ryegrass)
Allium cepa (common onion)
Daucus carota (carrot)
Zea mays (corn)

In addition, other species of economic or ecological importance to the region of impact may also be tested in lieu of these species. Two species are to be used in the plant uptake test.

These species have been selected for the following reasons:

- As food, forage, or ornamentals they are economically important and constitute major cash crops.
- Their distribution, abundance and taxonomic representation suggest broad coverage of the plant kingdom.

- They are also specified for phytotoxicity testing of pesticides (Subpart J, Pesticide Registration Guidelines). Additional justification for selection of these test species is provided in these guidelines (see FR 45(214): 72948-72978).
- They are sensitive to many toxic compounds and have been used to some degree in previous bioassays. Their use in herbicide bioassays, heavy metal screening, salinity and mineral stress tests and allelopathic studies indicates a sensitivity to a wide variety of stressors (Guenzi and McCalla 1966, Geronimo et al. 1973, Puerner and Seigel 1972, Wiedman and Appleby 1972, Reynolds 1978, Chang and Foy 1971).
- They are compatible with the environmental growth conditions and time constraints of the test method. Seed from the selected species germinate quickly and easily. Root growth is rapid and uniform. The seed contain no natural inhibitors and require no special pretreatment to germinate (such as soaking, chilling, prewashing, light, or scarification).

Other species may be substituted for any or all of these ten species when appropriate. For example, forest or desert species may constitute the population at greatest risk. If so, those of most value to man or of ecological dominance to the affected ecosystem should be selected. The rationale for selection of alternative species should be discussed with the Agency and/or supported in the report of findings.

2. Facilities

a. Apparatus

Greenhouse space, growth rooms, or environmental chambers are equally acceptable as long as the specified environ-mental conditions are maintained. The environment affects growth, metabolism, evapotranspiration, and photosynthesis and thereby directly and indirectly affects the plants' abilities to take up, metabolize, or accumulate chemicals. Laboratory facilities typically needed include standard laboratory glassware, a source of distilled water, work areas to clean and prepare equipment and to measure chemical concentrations and plant responses, drying ovens, appropriate equipment for chemical analysis, and proper disposal facilities. Without these facilities, the testing cannot be adequately conducted.

b. Containers and Support Media

Containers for hydroponic solution or root support media should be composed of inert materials, such as polyethylene, in order to prevent reaction of test chemicals and/or carriers with the container. The root support material should be composed of inert materials for these same reasons. Quartz sand and glass beads are suitably inert materials and do not readily adsorb substances. This lack of adsorption is important because the chemical should be available for root uptake; it would not be as readily available if it were adsorbed to the root support material or container wall.

Sand or glass beads are used, rather than soil, to avoid complications associated with variable physical and chemical properties and microbial populations indigenous to native

soils. Native soils are undesirable because of the varying clay, sand, and humus components, the types and proportions of which vary within the same soil type. Microbial populations also vary between soil types. These variables alter moisture holding capacity, chemical binding capacity, aeration, and nutrient and trace element content (Audus 1964, Beetsman et al. 1969, Beall and Nash 1969). In addition to the variations in these physical factors, there will also be variation in such chemical properties as pH and redox potential. Because of the impossibility of controlling physical and chemical properties of native soils, inert material is required to support the plants with the only variables being the presence and concentration of test chemical. The purpose of using glass beads or sand instead of native soils is not to make test results more directly applicable to natural systems, rather it is to eliminate sources of variation in the test.

c. Cleaning and Sterilization

Standard good laboratory practices are recommended to remove dust, dirt, other debris, and organic and inorganic residues from the test containers and support media which might confound test results. Residues could enter the hydroponic or nutrient solution and be taken up by plants, affecting their growth and/or other metabolic activity, resulting in misleading data. A dichromate solution should not be used for washing containers or beads because dichromate may enter the nutrient solution, be taken up by plants, and affect their growth and metabolic activity.

d. Nutrient Media

The nutrient medium specified in the protocol is modified half-strength Hoagland solution (Downs and Helmers 1975). This nutrient solution is recommended because all the constituent compounds are relatively easily obtained and because it works well for the culture of terrestrial plants.

Hydroponic or nutrient culture techniques eliminate spatial gradients within the growth medium and maintain the root system at uniform levels of aeration, nutrients, and water status (Rawlins 1979). The simplest and most practical method for routine plant growth in controlled environments is a sand or gravel culture in which the plants are grown in containers of sand which are periodically filled to provide water and nutrients, and drained to provide aeration. The frequency of irrigation depends on the storage capacity of the sand and the rate of water use by the plant. Use of aerated hydroponic solution without a root support media may be preferred by some laboratories.

Reliability is the primary consideration in the design of the system which automatically fills and drains the containers. One of the simplest systems includes a standpipe connected to an outlet at the bottom of each container through a manifold. The tops of the containers are at a uniform elevation. Periodically, the solution level in the standpipe is raised to the corresponding level and then lowered below the bottom of the containers. This system requires only one conduit to each container (for both filling and draining) and permits variation in the number of containers. The standpipe is filled by a pump from a lower storage reservoir. Electrical power to start the pump and

close the drain valve is controlled by a clock timer and, when the nutrient solution eaches the desired level in the standpipe, a float switch turns off the power to the pump and opens the drain allowing the solution to drain back to the reservoir.

If the rate of uptake for test chemicals applied in the nutrient solution differs from the rate of nutrient and water uptake, the exposure concentration will change with time and differ from that originally specified. In addition, chemicals applied to foliage as sprays or dust will enter the rooting zone and accumulate in the nutrient solution. While foliar application is designed to allow for chemical movement through, and uptake from, the rooting zone, excessive concentrations of test chemical should not be allowed to accumulate in the nutrient solution. The test solution should be replaced when or if the test chemical concentration differs from that specified by ± 20 percent. When the chemical is applied to the foliage, the nutrient solution should be replaced weekly if detectable quantities move through and accumulate in the solution following spraying or dusting of foliage.

3. Environmental Conditions

Controlled environmental conditions are necessary to maintain uniform growth and ensure reliable data. Maintenance of specified environmental conditions before, during, and after plant exposure to the chemical is essential to successful execution of this test. Variability of plant response to chemical exposure as a result of fluctuations in environmental conditions, has been noted (Darwent and Behrens 1972, Dunning and Heck 1973, Juhren et al. 1957, Leone and Brennan 1970).

Environmental conditions affect growth, metabolism, evapotranspiration, and photosynthesis of plants. In addition to mineral nutrition the conditions that should be standardized and maintained include: (a) carbon dioxide concentration; (b) relative humidity; (c) irradiation; (d) photoperiod; (e) day and night temperatures.

Standardization of environmental conditions is essential. Several investigators have demonstrated that differences in environmental conditions influence the response of plants exposed to chemicals. These include pre-conditioning by light and humidity (Dunning and Heck 1973), effects of temperature, photoperiod, and light intensity during the growth (Juhren et al. 1957), air movement during exposure (Brenann and Leone 1968), and mineral nutrition (Leone and Brenann 1970). Environmental conditions between growth chambers should be closely maintained, as specified in this protocol, to ensure common test conditions. If large growth rooms or greenhouse facilities are used, comparability of the environment between small groups of plants is not as critical, and environmental specifications may be relaxed.

a. Carbon Dioxide Concentration

The carbon dioxide concentration should be high enough for photosynthesis to occur at a level which will allow normal plant growth and biomass accumulation. Photosynthetic rates vary directly with carbon dioxide partial pressures. In addition, abnormally high levels of carbon dioxide can affect carbohydrate translocation from the leaves. Therefore, the concentration of carbon dioxide in the air surrounding test plants should be kept within

limits conducive to normal plant growth (350 ± 50 ppm). The carbon dioxide concentration is not expected to be of concern in greenhouse facilities.

In growth chambers, however, carbon dioxide depletion by rapidly photosynthesizing plants may be a real concern. The extent of depletion is a function of chamber volume, rate of air exchange between the chambers and the outside, the number, size, and type of plants, and the growth conditions maintained in the chambers (Hellmers and Giles 1979, Pallas 1979). Attempts to reduce carbon dioxide depletion by increasing the air exchange rate between the outside air and the chamber would probably eliminate temperature and humidity control within the chambers (Downs and Hellmers 1975). An appropriate system for carbon dioxide control in up to 5 chambers is described by Hellmers and Giles (1979). Alternatively, the use of fewer plants per chamber may be an appropriate means of avoiding carbon dioxide depletion problems.

b. Relative Humidity

A literature review by Hoffman (1979) indicates that most plants grow well when the atmospheric saturation deficit is maintained between 5 and 10 mb. At 25°C, the optimum relative humidity range is 65 to 85 percent. Most environmental control facilities with provision for humidity control can maintain humidity in this range with an accuracy at 25°C of ± 1.0 to 1.6 mb of vapor pressure (± 3 to 5 percent relative humidity). Humidity levels have been observed to affect plant growth and development, rates of carbon dioxide exchange, flowering, nutrient transport, and susceptibility to air pollution (Thurtell 1979). However,

these are generally the responses of plants to their total environments and are not unique to the humidity of the air. During the daylight period the relative humidity in the growth chamber should be maintained within the optimal range of 65 to 85 percent. When several chambers should be maintained as comparable environments, a relative humidity of 70 ± 5 percent is recommended. During the dark period, relative humidity should approach saturation, as it normally does in natural environments.

c. Irradiation

The growth chamber irradiation spectrum should be as similar to natural sunlight as possible in order for test plants to respond normally. Light drives the photosynthetic process and specific wavelengths cause particular responses (e.g., the phytochrome reactions) in plants. Therefore, it is important that light quality and intensity remain constant throughout the test. Irradiation in chambers should be 350 ± 50 uE/m^2 sec at 400-700 nm measured at the top of the plant canopy. This corresponds roughly to full, direct sun plus diffuse radiation. Artificial lighting is used in growth chambers and as supplemental lighting in greenhouses. However, lamps may weaken, cease to function, or function abnormally and fail in the course of long-term tests. Lamp failures should be corrected as soon as observed and the changes in light quality and intensity resulting from the lamp failures should be recorded.

For light quality and intensity to approach that of natural sunlight, the light source commonly consists of either a fluorescent-incandescent system, a high intensity

discharge system (HID) composed of metal halide lamps, or a combination of metal halide and high-pressure sodium lamps. Combinations of lamps are necessary for artificial lighting because there is no single lamp which is capable of emanating light of the same quality and intensity as natural sunlight, although the metal halide lamp closely approaches ideal lighting conditions.

HID systems produce intense light that may not be obtainable in other ways (McFarlane 1978). Because these lamps emit radiation in discrete line spectra, researchers frequently combine two different types of HID lamps in growth chambers to obtain a more balance spectrum. Metal halide lamps appear to be the most useful type of HID lamp because their emission spectra are almost continuous over the 400 to 700nm wavebands. However, a combination of metal halide and sodium HID lamps will provide superior plant growth to either one alone.

A fluorescent-incandescent system should be composed of 70-80 percent input wattage of cool-white fluorescent lamps and 20-30 percent wattage of incandescent lamps (Downs 1975). Although incandescent lamps are capable of emanating light which qualitatively approaches natural sunlight, the intensity will not approach the 350 ± 50 uE/m^2 sec specified for plant growth. Increasing the number of incandescent lamps would result in overheating the growth chamber. Fluorescent lighting alone can approach the intensity of natural sunlight without overheating the growth chamber, since very little of the output is in the infrared range (McFarlane 1978).

Incandescent lights should be included to provide radiation in the red and infrared regions. Uniformity and intensity of lighting within chambers is routinely improved by covering the walls with highly reflective materials.

d. Photoperiod

Plants exhibit three basic photoperiodic responses. Long-day plants will flower only when the light period is longer than a certain minimum number of hours in a 24 hour day/night cycle. Short-day plants will flower only when the light period is shorter than a certain maximum number of hours in a 24 hour day/night cycle. Day-neutral plants will flower regardless of the length of the light period. In this test, long day conditions (16 hours light/8 hours darkness) are recommended for all long-day and day-neutral plants in order to maximize biomass. Continuous light, however, is not recommended as a dark period is required for the phytochrome interconversions necessary in photo-synthetic vascular plants (Salisbury and Ross 1969).

e. Day/Night Temperature

For any particular plant species, there is an optimal temperature regime for maximum growth and development. This regime may differ between the phenophases of germination, vegetative growth, flowering, and fruit development. The concept of thermoperiodicity specifies that the temperature during the light period should be different from that of the dark period for optimal plant growth and development (Downs 1975). It has been demon-strated that plants grow and develop better with a day/night temperature differential (Kramer 1957, Went 1957). While specific temperature optima can be identified for each species, a regime of 25°C/20°C

± 3°C is recommended as it will promote suitable growth and be cost effective. If growth chambers or rooms are used, tolerances of ± 1°C are recommended to ensure environmental comparibility between growth facilities.

D. Reporting

The sponsor should submit to the Agency all data developed during the test. If testing specifications are followed, the sponsor should report that specified procedures were followed and present the results. If alternative procedures were used instead of those recommended in the test guideline, then the protocol used should be fully described and justified.

Environmental test (growth) conditions, chemical concentrations, quantity of chemical applied, number of applications, test data, and statistical analyses should all be reported. The justification for this body of information is contained in this support document. If plant species other than those recommended were used, the rationale for the selection of the other species should also be provided.

The data obtained from the plant uptake and translocation test should demonstrate whether or not there is uptake and translocation of the test chemical. Concentrations of substances in plants are commonly expressed in terms of weight per unit plant weight (e.g., ug/g dry plant). A subsample of plant material is selected after harvest and dried at 70°C for moisture content determination and expression of plant material on a dry weight basis. Concerns for chemical loss by volatilization in the drying ovens mandate that the entire mass of plant material not be dried. Concentrations of the test chemical

may be identical in two plants of different mass with the smaller plant containing less total chemical than the larger one. Test chemical concentration should be normalized with respect to mass in order to prevent the misleading reporting of uptake data. Both total quantity and concentration of chemical are required data.

If the chemical enters the root in bulk flow, transpiration and evaporation from the leaves will proportionately increase the uptake of the chemical. Plants that transpire more rapidly may have greater uptake rates. Consequently, a record of evapotranspiration rates is required. Chemical concentrations should be normalized with respect to evapotranspiration, as well as with biomass, in order to prevent misleading reporting of uptake data. Furthermore, any effects of the chemical on transpiration may also be identified.

The free parent chemical is that which is applied to the plant either in the nutrient media or as spray or dust and is extractable from the plant tissue in organic or aqueous solution. It is very important to quantify the free parent chemical as it is potentially toxic to living systems and appears in portions of plants consumed by man and domestic animals. Because of its potential toxicity, it is undesirable that such chemicals should be passed from one food web component to another. Data on the concentration of free parent chemical should be included in the report of testing because it probably is a measure of the maximum amount of chemical that can be expected to be found in plants exposed to the chemical in the natural environment. The quantity of chemical taken up by the plant is probably

more than would occur in the natural environment because the test is designed to provide data on "worst case" conditions.

The chemical may not remain intact in its original form in plants. Instead may be metabolized, combined with smaller or larger molecules, or otherwise modified. If the chemical or its degradation products enter normal plant metabolic pathways, metabolites may be formed. Since the chemical is not a normal substrate for metabolism, the metabolites are not likely to be those normally expected from plant metabolic activities. These abnormal metabolites may be chemicals that are as toxic or more toxic to living systems than the parent chemical. Although metabolite identification, because of analytical problems, is not the primary objective of the test, it may be possible to determine their presence in order to give some indication of chemical fate in plants and the mode of dissemination in food chains. This is especially important when the metabolites are potentially toxic. Bound residues, those forms of the test chemical which are not extractable in any of the solvents used, are portions of the chemical, or the intact molecule itself, which combine with structural components of plant cells. Bound residues may also be toxic to living systems because they may not remain bound when they are consumed and disseminated in food chains. Because of analytical problems, identification of bound residues is not an objective of the test; however, they should be quantified in order to give some indication of the fate of the chemical in plants and is mode of dissemination in food webs.

The total amount of chemical recovered in plants and nutrient media, after known addition of the chemical to the system, would indicate, by difference, the quantity lost by volatilization and/or degradation. Since the test from which these data are obtained is primarily an uptake test, mass balance data should be included to account for all the chemical added to the system as opposed to that which was actually taken up.

III. Economic Aspects

The Agency awarded a contract to Enviro Control, Inc. to provide us with an estimate of the cost for performing a plant uptake and translocation test according to this Guideline. Enviro Control supplied us with two estimates; a protocol estimate and a laboratory survey estimate.

The protocol estimate was $20,814. This estimate was prepared by identifying the major tasks needed to do a test and estimating the hours to accomplish each task. Appropriate hourly rates were then applied to yield a total direct labor charge. An estimated average overhead rate of 115%, other direct costs of $4,463, a general and administrative rate of 10%, and a fee of 20% were then added to the direct labor charge to yield the final estimate.

Enviro Control estimated that differences in salaries, equipment, overhead costs and other factors between laboratories could result in as much as 50% variation from this estimate. Consequently, they estimated that test costs could range from $10,407 to $31,221.

The laboratory survey estimate was $22,100, the mean of the estimates received from two laboratories. The estimates ranged from $19,200 to $25,000 and were based on the costs to perform the test according to this Guideline.

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EG-15
August, 1982

AVIAN DIETARY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

AVIAN DIETARY TOXICITY TEST

(a) Purpose. The guideline in this subpart is designed to develop data on the dietary toxicity to bobwhite and mallard of chemical substances and mixtures subject to acute environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003 15 U.S.C. 2601 et seq.). The Agency will use these and other data to assess the acute hazard to birds and to provide an indication of potential chronic hazard that these chemicals may present to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792--Good Laboratory Practice Standards apply here. In addition, the following definitions apply to this guideline:

(1) "Acclimation" Physiological or behavioral adaptation of test animals to environmental conditions and basal diet associated with the test procedure.

(2) "LC50" The empirically derived concentration of the test substance in the diet that is expected to result in mortality of 50 percent of a population of birds which is exposed exclusively to the treated diet under the conditions of the test.

(3) "Test substance" The specific form of a chemical or mixture of chemicals that is used to develop the data.

(4) "Exposure period" The five day period during which test birds are offered a diet containing the test substance.

(5) "Post-exposure period" The portion of the test that begins with the test birds being returned from a treated diet to the basal diet. This period is typically three days in duration, but may be extended if birds continue to die or demonstrate other toxic effects.

(6) "Test period" The combination of the exposure period and the post-exposure period; or, the entire duration of the test.

(7) "Hatch" Eggs or young birds that are the same age and that are derived from the same adult breeding population, where the adults are of the same strain and stock.

(8) "Basal diet" The food or diet as it is prepared or received from the supplier, without the addition of any carrier, diluent, or test substance.

(c) Test procedures--(1) Summary of test. (i) After birds have been obtained, they should be acclimated for at least seven days.

(ii) Test birds should be randomly assigned to the various treatment levels and controls.

(iii) Definitive test concentrations should be established, possibly requiring a range-finding test to be conducted first.

(iv) The test substance should be mixed thoroughly and evenly into the diet. Three treatment levels should be analyzed for test substance concentrations.

(v) Birds should be weighed at the beginning of the exposure period.

(vi) Birds should be observed regularly for mortality or abnormal behavior; any findings should be reported.

(vii) Food treated with the test substance should be replaced by untreated food (basal diet) after five days of exposure. Food consumption during the exposure period should be carefully estimated on a pen by pen basis.

(viii) Food consumption should be estimated for the post-exposure period and birds should be weighed at the end of eight days. Additional weights and food consumption estimates should be determined if the test period is longer than the typical eight days.

(ix) The mortality pattern should be examined, and a statistical analysis should be conducted. The LC50 slope, and confidence limits should be reported. A test for heterogeneity of data should be conducted.

(x) Treated or positive control birds should be sacrificed and disposed of properly. Negative control birds may be kept as breeding stock, but should not be used in any other tests.

(xi) The material to be tested should be analytically pure and the degree of purity should be reported along with the percentage of each impurity at levels specified in the test rule. If specifically required by a test rule for a particular substance or mixture, the technical grade should be tested. The test rule will specify the degree of purity or a range of compositions of the test substance.

(xii) A test is unacceptable if more than 10 percent of the control birds die during the test.

(2) [Reserved]

(3) Range-finding test. Unless the approximate toxicity of the test substance is known already, a range-finding test should be conducted to determine the test substance concentrations to be used in the definitive test, under paragraph (d)(4)(iii) of this section for details on concentrations for definitive tests. Procedures for range-finding tests may vary, but generally, groups of a few birds are fed three to five widely-spaced concentrations for five days. A concentration series of 5, 50, 500, and 5,000 ppm is suggested. The results of the range-finding test then may be used to establish the definitive test concentrations.

(4) Definitive test--(i) Controls. (A) A concurrent control is required during every test. The control birds should

be from the same hatch as the test groups. Control and test birds should be kept under the same experimental conditions. The test procedures should be the same for control and treated birds, except that no test substance should be added to the diets of control birds. If a carrier is used in preparation of the test diets, the same carrier should be added to the diets of control birds in the highest concentration used for test diets. The use of shared controls is acceptable for concurrent tests as long as the same carrier is used for all the tests.

(B) A test is not acceptable if more than 10 percent of the control birds die during the test period.

(C) A positive control (e.g., dieldrin standard) may be run, but is not required for each test. However, a quarterly or semi-annual laboratory standard (positive control) is recommended as a means of detecting possible interlaboratory or temporal variation. A laboratory standard is also recommended when there is any significant change in food, housing, or source of birds.

(ii) Number of animals tested. In the definitive test, a minimum of ten birds should be used for each dietary concentration of the test substance. A minimum of twenty birds should be used for the negative or carrier control. Thirty or more control birds are preferable. If a positive control or laboratory standard is used, ten or more birds should also be

used for each concentration of the positive control. When a test substance is known or expected to result in high experimental variation, it may be appropriate or required by the test rule to use additional birds.

(iii) Concentrations and dosage-mortality data. A minimum of five concentrations of the test substance should be used in the definitive test. These concentrations should be spaced geometrically. The recommended spacing is for each concentration to be at least 60 percent of the next higher dose (less than 1.67 times the next lower dose). If concentrations are spaced more widely than is recommended, then at least three concentrations should result in mortality between, but not including, 0 percent and 100 percent. For any concentration spacing, at least one concentration should kill more than 50 percent (including 100 percent) and at least one concentration should kill less than 50 percent (including 0 percent) of the birds in a pen. For some test substances, it may be necessary to use more than five concentrations to achieve these results.

(iv) Duration of test. The definitive test should include five days of exposure to the test substance in the diet (exposure period) followed by at least three days of additional observation (post-exposure period) while the test birds are receiving an untreated diet. If any test birds die during the second or third

day of the post-exposure period or if toxic signs are evident on the third day of the post-exposure period, the test period should be extended until two successive mortality-free days and one day free of toxic signs occur, or until 21 days after beginning the test, whichever comes first.

(v) Observations of record. (A) Throughout the test period, all signs of intoxication, other abnormal behavior, and mortality should be recorded and reported by dose level and by day. Signs of intoxication are those behaviors apparently due to the test chemical and may include a wide array of behaviors, such as labored respiration, leg weakness, hemorrhage, convulsions, ruffled feathers, etc. All signs of intoxication and any other abnormal behavior, such as excessive aggression, toe-picking etc. that may or may not be attributed to the test substance should be reported. Among survivors, remission of signs of intoxication and cessation of abnormal behavior should be recorded by dose level and by day. When differential signs of intoxication are observed within a dose level, an estimate of the number of birds exhibiting such signs should be recorded. Observation of test birds should be made, at a minimum, three times on the first day of the exposure period. Observations also should be made at least daily throughout the remainder of the test period; twice daily observations are recommended, where feasible.

(B) Average body weights of birds should be recorded and reported for each pen within each treatment and control group at the beginning of the exposure period and the end of the normal 3-day post-exposure period of each test. Body weights 72 hours before the exposure period are not required, but would provide valuable base-line data. Average food consumption should be measured in control pens and pens with the second lowest and second highest concentration levels either daily or every other day. Any significant amount of food spilled onto litter pans should be estimated and reported. For all other pens, average food consumption should be measured for both the exposure period and the normal 3-day post-exposure period. If the study is continued beyond eight days, body weight and food consumption data should be recorded weekly.

(C) Gross pathology examinations are not required, but they may provide valuable information on target site, mode of action, etc.

(5) [Reserved]

(6) Analytical measurements--(i) Statistical analysis. (A) A statistical analysis should be conducted by transforming the dietary concentrations to logarithmic values and the mortality pattern to probits. Other acceptable methods that will result in a theoretically straight line through $\pm$ 2 standard

deviations from the $LC50$ value may be used. The $LC50$ value and slope of the transformed concentration-resonse curve should be determined for mortality at the end of test period. Probit analysis by calculation or graphical probit methods are preferred. Any standard method that is used should provide the slope of the transformed concentration-response curve as well as the $LC50$ value. A statistical test for goodness-of-fit (e.g., chi-square test) also should be performed.

(B) All methods used for statistical analysis should be described completely.

(ii) Analysis for test substance concentrations. (A) Samples of treated diets should be analyzed to confirm proper dietary concentration of the test substance. Analyses should be conducted at the beginning of the exposure period with samples from high, middle, and low concentrations. If not already available, data should be generated to indicate whether or not the test substance degrades or volatilizes. If the test substance is known or found to be volatile or labile to the extent that 25 percent or more loss occurs over a five day period, then a second series of analyses of the same concentrations previously analyzed should be conducted at the end of the exposure period.

(B) The assay method used to determine actual concentrations should be reported.

(iii) Analysis of basal diet. A nutrient analysis of the basal diet should be included in the test report. For commercially prepared basal diets, the list of ingredients supplied by the company is normally sufficient if it is detailed. The composition of any vitamin or other supplements should also be reported.

(d) Test conditions--(1) Test species--(i) Selection. (A) Bobwhite, Colinus virginianus (L.), and mallard, Anas platyrhynchos L., are the test species. Birds may be reared in the laboratory or purchased from a breeder. If bobwhite are purchased, it is preferable that they be obtained as eggs which then are hatched and reared in the testing facility. During incubation, a temperature of 39°C and relative humidity of 70 percent are recommended for bobwhite. It is feasible to purchase live young bobwhite chicks if they can be obtained locally; however, young bobwhite may suffer adverse efects if shipped by air or other commercial means. Young mallard ducklings normally can be shipped without undue adverse effects.

(B) All control and treatment birds used in a test should be from the same source and hatch. Birds should be obtained only from sources whose colonies have known breeding histories. Birds

should be phenotypically indistinguishable (except for size) from wild stock. It is recommended that birds be obtained from flocks that have been outbred periodically with genetically wild stock in order to maintain a genetic composition that approximates the natural heterogeneity of the species.

(C) Birds used in the test should be in apparent good health. Deformed, abnormal, sick, or injured birds should not be used. Birds should not be used for a test if more than 5 percent of the total test population die during the 72 hours immediately preceding the exposure period. Purchased birds should be certified as disease free or as bred from disease free stocks. Birds should not have been selected in any way for genetic resistance to toxic substances. Birds should not have been used in a previous test, either in a treatment or control group.

(D) Test birds should be 10 to 17 days old at the beginning of the exposure period. All treatment and control birds in a test should be the same age $\pm$ one day. The exact age should be recorded and reported.

(E) Test birds should be acclimated to test facilities and basal diet for a minimum of seven days. Acclimation to test pens may be either in the actual pens used in the test or in identical pens. Birds used in the test should be assigned randomly to treatment and control pens without respect to sex. Randomization

may be done either at the initiation of the acclimation period or at the time when the birds are weighed at the beginning of the exposure period.

(F) During holding, acclimation, and testing, birds should be shielded from excessive noise, activity, or other disturbance. Birds should be handled only as much as is necessary to conform to test procedures.

(ii) Diet. (A) A standard commercial game bird (for bobwhite) or duck (for mallard) starter mash, or the nutritional equivalent, should be used for diet preparation. Antibiotics or other medication should not be used in the diet before or during the test. For bobwhite only, an antibiotic demonstrated to fully depurate in 72 hours may be added to the drinking water, if necessary, for birds up through 10 days of age; however, only clean unmedicated water should be offered during the 96 hours preceding the exposure period and during the test period. It may not be possible to obtain food that is completely free of pesticides, heavy metals, and other contaminants; however, diets should be analyzed periodically, and should be selected to be as free from contaminants as possible. A nutrient analysis and list of the ingredients in the diet should be included with the test report.

(B) The test substance should be mixed into the diet in a manner that will result in even distribution of the test substance throughout the diet. If possible, the test substance should be added to the diet without the use of a diluent. If a diluent is needed, the preferred diluent is distilled water; but water should not be used as a diluent for test substances known to hydrolyze readily. When a test substance is not water soluble, it may be dissolved in a reagent grade evaporative diluent (e.g., acetone, methylene chloride) and then mixed with the test diet. The diluent should be completely evaporated prior to feeding. Other acceptable diluents may be used, if necessary, and include table grade corn oil, propylene glycol, and gum arabic (acacia). If a diluent is used, it should not comprise more than 2 percent by weight of the treated diet, and an equivalent amount of diluent should be added to control diets for untreated birds.

(C) Diets can be mixed by commercial, mechanical food mixers. For many test substances, it is recommended that treated diets be mixed under a hood. Mashes and test substances should be mixed freshly just prior to the beginning of the test. For cetain volatile or other test substances, the Test Rule may require preparation of fresh diets at frequent intervals. Analysis of the diet for test substance concentrations is

required under paragraph (c)(6)(ii) of this section.

(D) Clean water should be available ad libitum. Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water should be changed at least once a day.

(2) Facilities. (i) Tests should be conducted with birds being maintained in commercial brooder pens or pens of similar construction. Pens should be constructed of galvanized metal, stainless steel, or perfluorocarbon plastics. Materials that are toxic, may affect toxicity, or may sorb test substances should not be used. Wire mesh should be used for floors and external walls; solid sheeting should be used for common walls and ceilings. Wire mesh for floors should be fine enough so as to not interfere with the normal movement of young birds. Pens for housing ten young birds should have a floor area of at least 3000 square centimeters (approximately 500 square inches) for bobwhite and 6000 square centimeters (approximately 1000 square inches) for mallards and should be at least 24 centimeters (approximately 9.5 inches) high. Pens should be disassembled (if feasible) and should be cleaned thoroughly between tests. Steam cleaning of cages is recommended. Cages may be brushed thoroughly, as an alternative method. The use of detergents or bleach is acceptable, but other chemical disinfectants such as quaternary

ammonium compounds should not be used. When necessary to control disease vectors, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended. Pens should not be cleaned during a test.

(ii) Pens should be kept indoors to control lighting, temperature, and other environmental variables. Pens should be heated, preferably by thermostatic control. A temperature gradient in the pen of approximately 35°C to approximately 22°C will allow young birds to seek a proper temperature. Temperature requirements for young birds typically decline over this range from birth through the first several weeks of life. Relative humidity is not as critical, but the test room should be maintained at a relative humidity of 45-70 percent. A photoperiod of 14 hours light and 10 hours dark is recommended. Other light/dark cycles should not be used, but continuous lighting is acceptable. Lighting may be either incandescent or fluorescent. Pens and lights should be positioned so that all pens will receive similar illumination. The facilities should be well ventilated.

(iii) Where feasible, it is recommended that pens not be stacked upon each other. If pens are stacked, only one test substance is allowed in any single stack. If a test substance

volatilizes or otherwise forms aerosols or vapors in the air, no more than one test substance should be tested in a room in order to avoid cross-contamination. Pens should be randomly arranged, whether or not in a stack, with respect to dose levels and controls. Pens, such as stacked, unmodified, commercial pens with external feeders, that allow food to be spilled from one pen to a lower pen, should be avoided. Any modifications that prevent cross contamination of concentration levels are acceptable. For example, commercially available, 30 cm (one foot) long chick feeders may be placed inside the pens and be covered with 1.27 cm (0.5 inch) mesh hardware cloth over the food, for bobwhite. The same feeders covered with approximately 2.5 cm (one inch) mesh wire are appropriate for mallards. For either species, external feeders can be covered with the appropriate size wire mesh and a solid piece of metal extended from the bottom of the cage to a point exterior to the feeder. Spillage may occur, but the added metal will prevent food from spilling into another feeder.

(3) [Reserved]

(e) Reporting. (1) The test report should include the following information:

(i) Name of test, sponsor, test laboratory and location, principal investigator(s), and actual dates of beginning and end

of test.

(ii) Name of species tested (including scientific name), age of birds (in days) at the beginning of the test, average body weights for birds in each pen at the beginning of the test, the end of the exposure period, and end of the test, and individual weights of all birds that die during the test.

(iii) Description of housing conditions, including type, size, and material of pen, pen temperatures, approximate test room humidity, photoperiod and lighting intensity.

(iv) Detailed description of the basal diet, including source, diluents (if used), and supplements (if used). A nutrient analysis of the diet should be included in the test report.

(v) Detailed description of the test substance including its chemical name(s), source, lot number, composition (identity of major ingredients and impurities), and known physical and chemical properties that are pertinent to the test (e.g., physical state, solubility, etc.).

(vi) The number of concentrations used, nominal and (where required) measured dietary concentration of test substance in each level, assay method used to determine actual concentrations, number of birds per concentration and for controls, and names of toxicants used for positive controls (if applicable).

(vii) Acclimation procedures and methods of assigning birds to test pens.

(viii) Frequency, duration, and methods of observation.

(ix) Description of signs of intoxication and other abnormal behavior, including time of onset, duration, severity (including death), and numbers affected in the different dietary concentrations and controls each day of the test period.

(x) Estimated food consumption per pen daily or every other day in the second highest and second lowest concentration and control pens. For other pens, food consumption should be estimated for the exposure period and for the post-exposure period.

(xi) Location of raw data storage.

(xii) Results of range finding tests (if conducted).

(xiii) The calculated LC50 value, 95 prcent confidence limits, slope of the concentration-reponse curve, the results of the goodness-of-fit test (e.g., chi-square test), and a description of statistical methods used. The same statistics for positive controls (when used). The methods used for statistical analysis should be described completely.

(xiv) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(2) In addition to the above information required in every report, the following information should be available upon request:

(i) A general description of the support facilities.

(ii) A description of the Quality Control/Quality Assurance program, including the Average Quality Level for the program element performing the test, procedures used, and documentations that these levels have been achieved.

(iii) The names, qualifications, and experience of personnel working in the program element performing the test, including the study director, principle investigator, quality assurance officer, as well as other personnel involved in the study.

(iv) Standard operating procedures for all phases of the test and equipment involved in the test.

(v) Sources of all supplies and equipment involved in the test.

(vi) Originals or exact copies of all raw data generated in performing the test.

ES-12
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

AVIAN DIETARY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TEST SUPPORT DOCUMENT FOR AVIAN DIETARY TEST

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-15 which uses Bobwhite and Mallard to evaluate the toxicity of chemical substances in the diets of these two species. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures, and conditions prescribed in the Test Guideline. Technical issues and practical considerations are discussed, In addition, estimates of the cost of conducting these tests are provided.

II. Scientific Aspects

A. General

Investigations of the dietary toxicity of chemicals to native birds began when the Fish and Wildlife Service was directed by Public Law 85-582 to evaluate and report upon the effects of pesticides on wildlife. As a result of this law, DeWitt et al. (1962) reported dietary toxicities of various pesticides to mallards and bobwhite exposed to treated diets at the Patuxent Wildlife Research Center (PWRC). Tests were conducted for less than 10 or less than 100 days with one objective being the determination of the quantity of pesticide producing at least 50% mortality within 10 or between 10 and 100 days (DeWitt et al. 1963). The shorter period was designed to simulate acute toxicity, whereas the longer period was to simulate chronic toxicity. Tests were conducted with adult and/or young bobwhite, mallard, and ring-necked pheasant.

Similar studies were conducted at PWRC in 1963 by Stickel (1964), who reported at this time that young birds used in dietary tests were 1-2 days old. Japanese quail were also used as a test species beginning in 1963. The 1963 tests were from 3 to 185 days in length.

Heath and Stickel (1965) presented a protocol for avian dietay tests. This protocol recommended a test period of 5 days exposure to a treated diet followed by 3 days of observation while the birds received untreated diets. The standardized length of the test period was designed to permit quantitative comparisons of the relative toxicities of different test substances. This protocol also recommended that the birds be 5-7 days in age when first exposed to treated diets.

This protocol was generally followed by PWRC for 10 years, although some deviations were occasionally necessary because of shortages of facilities or birds (Hill et al. 1975). Hill et al. (1975) reported that the ages of the test birds were standardized in 1970. Quail (both bobwhite and Japanese quail) were tested when they were 14 days old; mallards and pheasants were 10 days old. In 1973 tests, mallards were five days old. Heath et al. (1972) did not specify the ages of test birds precisely, but they did state that dosage was never initiated before birds were nine days old.

EPA adopted the basic protocol described by Heath and Stickel (1965) and Heath et al. (1972) and published it as a test method to be used in conjunction with pesticide registration (US EPA 1975). The EPA protocol was intended primarily for bobwhite and mallard and specified the use of

10-15 day old birds. In other respects, the EPA protocol generally followed that developed by PWRC. The Agency (US EPA 1978a) published a later protocol that was slightly modified from the first EPA protocol. This later protocol specified, as a guideline, that birds be 10-17 days old at the beginning of the test period and also recommended a continuous lighting regime. Other modifications were minor.

Most avian dietary tests conducted since 1965 have more or less followed either the Heath and Stickel (1965) protocol or one of the EPA protocols. The largest number of avian dietary tests have been conducted for the purpose of pesticide registration and have been classified as confidential business information by the sponsors. PWRC has conducted the vast majority of avian dietary tests that have been published in the open literature (Heath et al. 1972, Hill et al. 1975). Very recently, ASTM (1979a) has developed a draft protocol for avian dietary tests that also is based on the PWRC method.

Because all known avian dietary test protocols are based upon the method of Heath and Stickel (1965), there has been no need to compromise between widely divergent methods. At the same time, there have been numerous deviations from the basic method that preclude precise comparisons of all of the data. There continue to be conflicts of ideas among investigators conducting avian dietary tests. Yet, there are few published data to support either side of many conflicts. Also, there is little ongoing research that might resolve these conflicts. PWRC does conduct methods research to some extent, but reports from most other avian dietary test facilities usually are classified as confidential.

To promote uniformity and comparability of tests, some recommendations and requirements are standardized in this test guideline. Where such recommendations and requirements are controversial and are not addressed sufficiently by published data, it is hoped that research will be stimulated to resolve questions. If feasible, conditions and procedures that approximate natural conditions have been specified in preference to other options. Controversial points are addressed in section 1.2 of this support document.

The use of avian dietary tests in the assessment of chemical impacts on the environment is based on several factors. First, birds are an obvious and important component of the environment. Congress has indicated repeatedly that birds are worthy of protection by passing such laws as the Lacey Act of 1900, Migratory Bird Treaty Act of 1918, Migratory Bird Conservation Act of 1929, Pittman-Robertson Act of 1937, Fish and Wildlife Act of 1956, Endangered Species Act of 1973, and others. The United States also has entered into treaties with Great Britain and Canada (1916), Mexico (1937), Japan (1974), and Russia (1976) for the protection of migratory birds. The people of the United States have also indicated a desire to protect birds through their support of Audubon Society, Nature Conservancy, and other environmental groups. Sportsmen's organizations support protection of birds, although their interests often focus heavily on game birds.

Second, birds have a definite economic importance. Federal and State Agencies spend large sums for the preservation and propagation of birds. Hunters and

birdwatchers also spend substantial sums in pursuit of their pastimes. Less measurable, but of definite importance, is the substantial role of birds in insect control.

Birds consume huge quantities of insects and other invertebrates, many of which are considered pests. Small mammals and other vertebrates or plants are consumed by various birds, sometimes to the extent that birds have an important effect on populations. In turn, birds are consumed by birds of prey, mammals and other vertebrates. Excretory products of birds provide nutrients for plankton and other microorganisms that in turn are food for larger organisms. Birds are important in pollination of some plants and in dispersal of others. Because of their mobility, the effects of or on birds are not restricted to specific locations.

Finally, birds are among the more sensitive terrestrial vertebrates. Because of their high metabolic rate, high body temperature, and the demands of flight, they require more energy relative to their size than most other animals. The energy requirements lead to greater food intake and thus to greater toxicant intake when a toxicant is in or on their food. Data presented by Kenaga (1979) indicate that for a majority of insecticides, mallards are more sensitive than rats in acute oral tests. No data were presented for acute oral toxicity to bobwhite, but in dietary tests, bobwhite are typically more sensitive than mallards (Heath et al. 1972). These data and confidential reports submitted to the Agency for pesticide registration suggest that, in general, birds are the most sensitive class of terrestrial vertebrates.

Avian dietary tests are useful in assessing the hazard and risk of toxic substances to avian species. They are relatively inexpensive and of short duration. Since ingestion is the most likely route of avian exposure, dietary tests can be used to estimate the effects of short-term exposures. When a concentration-response curve is generated, as is required in this guideline, the curve can be used to estimate a probable no-effect level and to obtain indications of chronic effects. The results can be used to compare toxic responses between or among species or of one species to various test substances. The results of laboratory dietary studies can be extrapolated to field conditions and/or to other species. All such comparisons and extrapolations should be made with extreme caution, taking into account a number of physiological, ecological, and behavioral parameters.

Avian dietary tests are different from avian acute oral tests. Acute oral tests involve a single dose, usually administered by capsule or gavage directly into the crop. Amounts are varied according to the weight of the bird, so that each bird receives the same dose on a mg/kg basis. Dietary tests, on the other hand, involve ingestion of a test substance that is incorporated into the diet. Because it is not practical to house birds in individual cages, food consumption, and thus test substance intake, cannot be measured individually but rather are determined on an average basis for each pen. Some birds may eat more or less than the average. In addition some food is spilled, and some birds may reduce consumption because of aversion to the food and test substance or because of toxic effects of the

test substance that they have ingested. Therefore, the precise amount of test substance taken in cannot be determined and may vary among the test birds. As a result, the LC_{50} values determined in dietary tests are not as precise as LD_{50} values obtained in acute oral tests. However, dietary tests do simulate exposure under natural conditions. They take into account not only the route of exposure, but also individual variation in intake, aversion, reactions to test substance and, to a limited extent, detoxification of the test substance. Acute oral tests have definite value, particularly in comparative toxicology or in assessing hazards of such concentrated toxicants as granular insecticides. But it is the dietary test that is most useful in assessing the hazard and risk of most test substances to birds.

1. Issues

The avian dietary test guideline and support document contain some controversial points. Data are insufficient or absent to support either side of most points. For other points, there are data to support each side, or the controversy may relate to the use of the test. Issues are merely identified below and are discussed in the appropriate sections of this document.

- Is continuous lighting more appropriate than dark/light cycles, or should either be considered acceptable?
- What is the most appropriate age for test birds? How narrowly should the age be defined?
- Is a positive control group(s) necessary for each test?

- o What carriers, if any, should be used or allowed for incorporation of the test substance into the diet?
- o Are commercial foods adequate, or is there too much variation and/or contamination of commercial foods?
- o Should treated diets be mixed daily with the same or decreasing concentrations, or should diets be mixed fresh only at the beginning of the test?

B. Test Procedures

1. Range Finding and Definitive Dose Levels

The range-finding test is a highly recommended procedure (US EPA 1978a, ASTM 1979a) which helps to determine the appropriate dietary concentrations in the definitive test. In some situations there may be enough toxicity information available so that appropriate concentrations can be selected without a range-finding test. The range-finding test (or other available information) needs to be accurate enough to ensure that dose levels in the definitive test are spaced so as to result in at least one level each above and below the LC_{50} value.

Unless otherwise specified in the Test Rule, it is necessary to establish dose levels in the definitive test that will result in an accurate determination of the slope of the log dose-probit response curve. The slope and shape of this regression curve are of great interpretative value in analyzing the results of the test (Tucker and Leitzke 1979). Among other things (refer to discussion on statistical interpretation), the slope and mortality pattern may yield an indication of chronic effects. A shallow slope suggests chronic effects whereas a steep slope suggests

acute effects (Tucker and Leitzke 1979). Because this avian dietary test directly investigates short term effects, it is more important that a shallow slope be well defined than it is for a steep slope. A slope can be defined well by spacing concentrations to yield several partial mortality levels, although individual variation in response, for any reason, will tend to reduce precision of results. Ideally, three or more partial mortality levels will give a reasonable indication of the precision and accuracy of the slope. Therefore, close spacing of concentrations is recommended to yield these partial mortality levels. When a spacing factor of 1.67 is used, as is recommended in the guideline, and does not result in three partial mortality levels, then the slope is steep, usually greater than 6 probits per log cycle. As stated above, a steep slope does not need to be completely defined because the test itself predicts acute effects. Thus, it is not necessary to use closer spacing than a 1.67 ratio. However, if spacing is wider than recommended, then it is necessary to achieve three partial mortality levels in order to yield a reliable slope.

The requirement for at least five concentration levels is also based on the need for at least three partial mortality levels to provide for reliable probit or other statistical analysis. In the event that five concentration levels provides five partial mortality levels, this situation would yield an even more reliable slope and LC_{50} value. Fewer than five levels may frequently yield sufficient information for determination of an LC_{50} value by non-parametric statistical methods, but even more

frequently, fewer than five levels will not yield the three partial mortality levels necessary for an accurate slope determination.

Some test substances are relatively non-toxic and are unlikely to pose an acute environmental problem. In terms of acute effects, the Patuxent Wildlife Research Center (Heath et al. 1972, Hill et al. 1975) and the Agency's Office of Pesticide Programs (US EPA, 1978a) have both stated that it is unnecessary to determine an accurate dietary LC_{50} when it is found to be in excess of 5000 ppm. Some test substances may have an LC_{50} greater than 5000 ppm, but may still produce mortality at the 5000 ppm level. Since the slope of the log dose-probit regression line is as important as the LC_{50} value in interpreting the results of a test, when mortality does occur at 5000 ppm, it is important to determine at what level there is no observed effect. If the no effect level is comparatively low, such as around 500 ppm, this gives an indication of the potential for chronic toxicity and the need for additional testing. A comparatively high no effect level, such as 4500 ppm, suggests a steep slope and considerably less possibility of chronic effects. In essence, the mortality at 5000 ppm and the no effect level give a rough indication of the slope and therefore, range-finding dose levels of 5, 50, 500, and 5000 ppm are suggested in order to incorporate the 5000 ppm level (relatively non-toxic) and a lower no effect level in case there is some mortality at 5000 ppm.

2. Controls

Concurrent controls are required for every test to assure that any observed effects are a result of ingestion

of the test substance and not of other factors. Other factors may include such environmental factors as temperature, lighting, vapors, sensitive or stressed test birds, etc. If a diluent is used in mixing the diet, this diluent is used also in the untreated diets at the same concentration as it occurs in treated diets. In effect, this results in a diluent, but no completely negative, control. Diluent selection is based upon an assumed lack of toxicity (e.g., water, completely evaporated acetone) and it is not considered necessary, therefore, to have an additional negative control when a diluent control is used.

A positive control, such as one with dieldrin, is recommended as a means of detecting temporal or interlaboratory variation. Some researchers have advocated using a positive control for every test and then adjusting the test substance LC_{50} value by dividing that value by the positive control LC_{50} value to yield a ratio that can then be used to compare toxicities of various test substances (ASTM 1979a). This procedure may be of academic interest when comparing different test substances, but it is inappropriate for the purpose of this guideline. In addition, the mathematical manipulation of two values that both have limits on precision as indicated by confidence intervals will result in a further decrease in precision when the results are combined. Another argument has been advanced (Heinz, personal communication) that a positive control should still be run with every test, even if no adjustments are made. It is assumed that the procedures outlined in this guideline are rigorous enough so that variation within a given laboratory will be minimal as long

as the food, cages, and source of birds remain the same. A quarterly or semi-annual positive control, or one performed when the food, housing, or source of birds changes, should provide enough information on temporal or interlaboratory variation to preclude the need to have a positive control with every test.

3. Number of Animals Tested

Ten birds per test concentration is the minimum number required in this test. Other test methodologies have recommended ten birds per level but have allowed as few as six birds per level (ASTM 1979a, US EPA 1978a). For some, perhaps many, test substances, six birds per level will yield statistically valid results. However, the statistical error of variable responses will be magnified when six birds are used, and a significant number of tests may result in values that are not statistically valid.

It also should be noted that the protocol recommended by the Agency requires no less than ten birds per dose level in avian acute oral tests required for pesticide registration (US EPA 1978b). Since the acute oral tests inherently control more variables than dietary tests (see section 1), it would be inconsistent to allow fewer birds in the dietary tests than in the acute oral tests. The vast majority of avian dietary tests have been conducted either by Patuxent Wildlife Reseach Center (Heath et al. 1972, Hill et al. 1975) or by contract and company laboratories for the purpose of pesticide registration. Most of these tests have utilized ten birds per level and only a small fraction have used less than ten. These facilities apparently favor the use of ten birds per level because it markedly reduces the

number of tests that have to be rerun to achieve statistical validity and because it is nearly as easy to maintain ten birds in the appropriate size pen as it is to maintain six birds in the same size pen.

Ten birds per concentration usually are adequate to produce a valid test, although some test substances may require more birds to accommodate excessive variation in response. Although 20 birds per level may be considered statistically superior to ten birds per level, there is a substantial data base for using the latter, and most tests using 10 birds per level have achieved statistical significance. For the small number of tests that resulted in heterogeneous data, it is more cost-effective to repeat a test with more birds per level than it is to require all tests to be conducted with more birds. The use of 20 birds per dose level would require twice the pens, space, food, care, handling, etc. and would significantly increase the cost of a test. Not only would the cost be increased, but due to limited avian testing facilities, the time between requiring a test and beginning the test would be lengthened (on the average) if 20 birds per concentration were required. Some types of tests, such as aquatic tests, may require 20 organisms per level to offset the greater number of variables involved or the variable responses of the test animals. It is not necessary that avian tests also require 20 birds per dose level.

Twenty birds are the minimum required, however, for negative control groups. Many investigators use the same number of control birds as are used in all experimental levels tested, and this number (50-60) is required in one

method (ASTM 1979a). Frequently, such a large control group is used for several concurrent tests. In such a situation a failure to achieve a control survival of 90% would mean that all of the concurrent tests would be unacceptable. In addition, 20 control birds, or preferably 30 or more, will provide better data on food consumption and growth values with which to compare performance of treated birds. Although natural mortality of young birds usually occurs prior to reaching the test age, natural mortality during the test may occur, more often with bobwhite than with mallards. Since control mortality in excess of 10% invalidates a test, the use of 20 birds in a control will nearly always yield sufficient survival for validity. The use of a single extra pen of 10 control birds is a cost-effective preventive measure that in addition yields a statistically more powerful test.

4. Duration of Test

Acute toxicity tests with birds and mammals are normally done on an oral single dose basis and historically the single dose has been followed by 14 or 21 days of observation. This was originally true of laboratory mammals and also was adopted by those investigators conducting acute single dose oral tests on birds (Tucker and Crabtree 1970). Avian dietary tests, however, have developed along other lines. The duration of the test was first standardized by the Patuxent Wildlife Research Center (Heath and Stickel 1965). The test period was set at 5 days dietary exposure to the test substance followed by 3 days of observation while birds were receiving untreated diet. Using the PWRC format as a basis, the Agency's Office of

Pesticide Programs (US EPA 1978a) and subsequently ASTM (1979a) both presented test methods with the same duration. Because of this historical basis and the data base developed from these methods, the duration of the currently proposed dietary test is the same.

The three day post-exposure observation period was originally included to detect chemical mortality induced beyond the exposure period (Heath et al. 1972). It was noted that one chemical caused a delayed mortality pattern, and for that chemical, the observation period was extended an additional six days. Subsequent adaptations of this protocol (US EPA 1978a, ASTM 1979a) included statements that the observation period "shall be extended...as long as test birds exhibit toxic symptons and continue to die." In tests submitted to support pesticide registration, however, it has been noted that tests were rarely extended beyond eight days, even when mortality occurred on the last day. It was, therefore, determined that the currently proposed avian dietary guideline should be more definitive about the length of the post-exposure period under certain conditions. It is not expected that the post-exposure period will need to be extended for most test substances.

5. Observations and Measurements

When birds are caged together, the amount of food ingested cannot be determined for individual birds. In addition, feed can be spilled out of the pen or scattered among litter and droppings. Therefore, only estimates of average consumption per pen can be determined. This admittedly does not yield precise intake data per bird, but the dietary test is considered an "applied" measurement that

includes factors of ingestion and digestion in addition to toxicity (Heath et al. 1972).

It would be desirable to have the body weights of test birds recorded at the beginning of the test, at the end of the exposure period, and at the end of the test. However, weighing birds at the end of the exposure period requires handling, and the stress on the birds, especially in combination with test substances that produce stimulant effects, could easily lead to anomalous results. Therefore, body weights are required only at the beginning and at the end of the test.

Observations of signs of intoxication are important for several reasons. First, they obviously yield information on the mode of action of the test substances. If there is additional information from other sources on the mode of action, signs of intoxication other than those known indicate either a difference in action between the other test animals and birds or that factors other than the test substance are involved. The duration of signs of intoxication may yield information on the potential for cumulative effects or the possiblity that effects are irreversible.

6. Required Analyses

a. Statistical Analysis

A coherent theory of the dose-response relationship was introduced by Bliss (1935), and is accepted widely today. This theory is based on four assumptions:

- Response is a positive function of dosage, i.e., it is expected that increasing treatment rates should produce increasing responses.

- Randomly selected animals are distributed normally in Gaussian fashion as to their sensitivity to a toxicant.
- Due to homeostasis, response magnitudes are proportional to the logarithm of the dosage, i.e., it takes geometrically increasing dosages (stresses) to produce arithmetically increasing responses (strains) in test animal populations.
- In the case of direct dosage of animals, their resistance to effects is proportional to body mass. Stated another way, the treatment needed to produce a given response in a population of test animals is proportional to the size of the animals treated.

If percent mortality is plotted as a function of the logarithm of test solution concentration, the form of the resulting curve will generally be sigmoidal (Casarett and Doull 1975). The sigmoidal shape of the concentration-response curve makes it less than ideal for predictive purposes. It is often more useful to transform the data so that the concentration- response curve may be represented by a linear equation of the form:

$$y=ax+b$$

While a number of data transformations are possible, the probit or probability unit transformation (Bliss 1934) is the most widely accepted means of linearizing the sigmoidal concentration-response curves (Finney 1971).

Probits are standard deviations to which the number five has been added arbitrarily to avoid negative numbers. For

example, the 50 percent response point is equivalent to the median and is called probit five. An 84 percent response is the median plus one standard deviation and is also known as probit 6 (0+1+5=6). Likewise, a 16 percent response is the median minus one standard deviation or probit 4 (0-1+5=4).

Once the mortality data have been transformed onto a probit scale, a straight line can be fitted graphically according to the methods of Litchfield and Wilcoxon (1949) or Miller and Tainter (1944) or by calculated methods such as that developed by Finney (1971). The LC_{50} value can be determined from the regression line, and the 95% confidence limits can be calculated. Other values (e.g., LC_{16}, LC_{84}) also can be determined from the line along with the corresponding confidence limits. However, the confidence limits are narrowest at the LC_{50} value and wider as the LC_x value is further from the LC_{50} point.

The LC_{50} value itself has utility in that it provides an indication of the toxicity of a test substance. This value can be used to help predict the likelihood of short-term adverse effects to animals exposed to the test substance in the environment. It should be noted, however, that the endpoint of this test is mortality and the exposure period is 5 days. Thus, the LC_{50} value is most pertinent to relatively short term effects.

The mortality pattern and slope (probits divided by log cycles) of the concentration-response line have more implications. A low (<2 probits/log cycle) slope or delayed mortality is usually indicative of slow and often cumulative effects, whereas a steep slope (> 6 probits/log cycle) suggests rapidly acting effects (Tucker and Leitzke 1979).

When the effects of a test substance are slow and cumulative, there is a strong possibility that some effects may occur at concentration levels well below the LC_{50} value and also that sublethal effects have a higher likelihood of occurring in many animals. When a toxic substance accumulates in an animal at sublethal levels, there is a likelihood that chronic effects, such as reproductive impairment or secondary toxicity, may occur. Significant amounts of some toxic accumulative chemicals, such as DDE and heptachlor epoxide, are excreted via the eggs of birds (Stickel, 1973) and may adversely affect reproductive success. Thus, a low slope or delayed mortality that suggests a potential for accumulation and chronic effects may have long lasting consequences for the populations of exposed birds. Therefore, not only the placement, but also the mortality pattern and slope of the concentration-response line are important pieces of information derived from this test.

b. Test Substance Concentrations

Samples of treated diets will be analyzed to determine the actual levels to be used in the test. Analysis will help to detect mathematical errors in calculating concentrations, technicians' errors in mixing diets, and manufacturers' errors in determining the amount of active ingredient in a test substance (Heinz, personal communication). Intentional manipulations of dietary concentrations will not normally be detected simply through analysis, unless it can be ensured that those diets analyzed are those diets actually used. Therefore, it is sufficient to analyze dietary concentrations at several, rather than

all concentration levels, with the assumption that variations between measured and nominal concentrations will be consistent for all levels. When volatile or labile test substances are used, it is important to analyze concentrations at the end of the exposure period also.

c. Basal Diet

Most testing facilities use diets prepared by commercial feed companies. Some facilities may have a commercial company prepare a diet to order. Normally, such diets are supplied with a quantitative list of ingredients, and such a list should be supplied with the test report. If there are supplements added to the diet, a list of all supplemental ingredients also should be submitted. Analysis of ingredients in the basal diet is important because there are a number of potential test substances, such as certain metals, that may interact with components of the diet and possibly affect the results of a test. A nutrient analysis will allow for a better evaluation of such results. In addition, it is possible that dietary deficiencies or imbalance of ratios of nutrients also could affect the results. Even though commercial companies normally supply a nutritionally adequate diet, it is important to know the components because no rigid requirements exist for the type and constitution of the diet used.

7. Acceptability Criteria

A typical avian dietary test will have no control mortality. Occasionally, a bobwhite control death may occur, but only rarely will a mallard control death happen. In well conducted tests, control mortality will never ($p > 0.99$) be greater than 10%. If control mortality

exceeds 10%, it is very likely that it is due to improper test conditions or procedures, such as failure of temperature control, excessive handling, etc. It can be assumed that factors causing control mortality will also contribute to mortality of treated birds and result in inaccurate results. Therefore, a test is considered unacceptable if more than 10% of the control birds die.

C. Test Conditions

1. Test Species

a. Selection

The mallard, Anas platyrhynchos, and/or bobwhite, Colinus virginianus, are the species to be tested. The choice of these species is based on a number of factors. Although no single species would satisfy all criteria for species selection, mallard and bobwhite each have a number of favorable attributes.

The mallard has a widespread distribution, not only in the United States, but also in Eurasia. Such distribution means that mallards may be exposed to toxic substances in the environment regardless of the location of the toxic substance. As a waterfowl, mallards may also be exposed to toxic substances in the water, in sediments, and on land. Because the mallard is the most abundant and widely distributed duck in the northern hemisphere (Bellrose 1976), it is also suitable as a native test species for many countries belonging to the Organization for Economic Cooperation and Development (OECD).

The bobwhite has a widespread distribution throughout much of the United States, and it is an important part of the avifauna of the southern U.S. It occurs in a variety of

errestrial habitats and is likely to be exposed to toxic ubstances that occur in such habitats. Because it is idely distributed, the bobwhite represents itself for many nvironments as well as serving as a surrogate for other errestrial avian species.

Bobwhite and mallards also are amenable to testing in he laboratory. They can be bred in captivity and are eadily available from commercial sources so that testing of hese species will not deplete wild stocks. There is ufficient information on the nutritional, habitat, and ehavioral characteristics of natural populations of allards and bobwhite in order to meet the basic nutritional nd physical requirements of the species in the laboratory.

Mallards and bobwhite have been demonstrated to be ensitive to challenges with toxic chemicals (Tucker and rabtree 1970, Hill et al. 1975). In a comparison of four pecies (mallard, bobwhite, Japanese quail, ring-necked heasant) that all were tested with the same 39 chemicals in ietary studies, bobwhite was the most sensitive to 47.4% of he chemicals (Heath et al. 1972). Even though this omparison showed bobwhite to be the species most sensitive o the largest number of toxic chemicals, relative species ensitivity is variable depending upon the particular test ubstance or type of test substance. Therefore, dietary ensitivity, an important criterion, should not be the verriding factor in selection of species for these tests. or example, waterfowl have demonstrated a particular usceptibility to eggshell thinning in reproductive studies nd bobwhite have been shown to be sensitive to the eproductive effects of a few chemicals such as toxaphene

(DeWitt et al. 1962). Both species have been widely used in actual and simulated field tests. It is advantageous to select for short term tests a species which also can be used in reproductive and field tests. This allows for more direct comparisons between the results of two test types than can be obtained when one species is tested in short-term tests and another species is tested in reproductive or field studies.

In addition, mallards and bobwhite are generally considered to have a positive economic value. Although the Agency is charged with the protection of all species in the environment, the choice of an economically valuable species for testing is appropriate to the cost-benefit or risk-benefit analyses upon which Agency decisions are frequently based.

Finally there is as good or better a data base for toxicity tests with mallards and bobwhite as for any other native avian species. This data base permits comparisons with results of tests with other toxic substances.

If a test is to simulate toxicity to naturally occurring populations of mallard or bobwhite, then it is important to use birds that are phenotypically indistinguishable from wild birds. Since many chemicals act upon specific enzymes and enzymes are based on a genetic code, the use of birds genotypically similar to wild birds would be desirable. However, the determination of phenotype is a simple observational process, whereas genotypic determination is impractical, if possible at all. In addition, wild birds have a degree of heterogeneity that would not be typical of any given genotype.

The necessity for using healthy birds is obvious since the test is designed to determine toxicity to healthy, typical populations. The health of birds also is important in reducing the number of variables that limit comparisons between tests. There are several checks in this test guideline that help to ensure that healthy birds are used. The use of previously untested birds not selected for resistance and being from disease-free flocks provides a basically healthy stock. Visual observations select out apparently abnormal or unhealthy birds from that stock. A final check on health is based on the birds' ability to survive the three-day period preceding the test. The 5% maximum mortality during this period, as established by the Committee on Methods (1975) for effluent fish tests and as proposed by ASTM (1979a) for avian dietary tests, allows for the natural mortality that may occur in young birds, but precludes the use of populations that do not demonstrate a reasonable survival rate.

Historically, birds used in dietary tests usually have been immature birds. Hill et al. (1975) reported dietary toxicities of pesticides to four avian species. All birds were between 5 and 24 days old, with 85% being 10 to 17 days old. The only significantly repeated deviation from the 10 to 17 day age group was that 15% of the mallards were aged 5 days. Guidelines for the Agency's Office of Pesticide Programs specify using 10 to 17 day old birds (US EPA 1978a). A large number of avian dietary tests have been conducted with this age class, although these data are confidential and generally not available to the public. Because of the historical precedents and substantial data

base for mallards and bobwhite of this age, 10 to 17 day old birds are specified.

Certain investigators (Heinz et al. 1979) have argued that mallards should be tested when they are five days old. This has been proposed because five day old mallards could not survive the test period if they refused to eat and because it is thought that the younger birds are more sensitive. However, it is felt that the death of birds in a test from starvation does not approximate natural conditions because wild birds are rarely restricted enough to have only one food source. Further, death by starvation confounds the interpretation of LC_{50} values. With many chemicals, younger birds do appear to be more sensitive, but age sensitivity is not constant, even for a given class of chemicals (Hudson et al. 1972). Because of these factors, since adult birds are more likely to be exposed to toxic chemicals than are young birds, and since use of younger birds is likely to result in increased control mortality, there appears to be no compelling argument to change the historical precedence and use 5 day old mallards.

Randomization of test birds into the test cages prevents biases from being introduced. Most test birds are artificially incubated and subtle differences in incubators can result in hatchlings from one part of the incubator having different characteristics, such as body weight, from hatchlings in another part of the incubator. Randomization is done without regard to sex because young birds cannot be sexed reliably without surgery. Since all test pens are supposed to be treated equally in a manner to reduce variables, it is not necessary to acclimate the birds in the

actual pens used in the test. Birds are normally handled after hatching or upon receipt and again when they are weighed at the beginning of the test period. Randomization at either of these times is preferable to handling the birds an additional time simply for the purpose of random assignment.

2. Maintenance of Test Species

a. Acclimation

Acclimation of birds to test cages and basal diet is important so that the effects of a new environment on the toxicity of test substances are limited. Ideally, birds should be maintained in test cages and on basal diet from the time of hatching until the test period, However, many test facilities purchase young birds rather than rear them from eggs. A seven day acclimation period allows enough time for adaptation and permits these facilities to purchase birds if such facilities cannot rear their own.

b. Diet

There are few data on the nutritional requirements of mallards or bobwhite. This subject is being investigated under a current contract and is a proposed research need. At the present time, commercial starter mash is recommended, based upon historical precedents (Hill et at. 1975, US EPA 1978a, ASTM 1979a) and a lack of data that support alternatives. Changes may be made in the future when additional data become available.

There are several different ways of considering the test concentrations with respect to degradation and actual amount of test substance present. One view is that the test substance should be mixed freshly with the diet every day so

that a constant level is achieved. A second is that the diets should be mixed only at the beginning of the test in order to simulate natural degradation in the environment. A third view holds that diets should be freshly mixed each day, but in decreasing concentrations that simulate natural degradation. The second alternative above has been used in most avian dietary tests in the past. Although degradation rates in food may differ from rates in the environment, there are differences in environmental degradation rates among the various compartments (soil, on plants, in plants, etc.). If there were a single degradation rate, the third alternative would offer advantages, possibly enough advantage to counterbalance the additional cost of mixing diets daily. The first alternative is more costly and would not simulate natural conditions unless release of the test substance was continuous. Since the history and previous data are based on the second alternative and since this alternative does simulate degradation to an extent, the test guideline specifies that diets should be mixed once at the beginning of the test (unless otherwise specified in the Test Rule). If degradation is expected to be substantial (greater than 25%), then concentrations should be analyzed at the end of the exposure period in order to ascertain the extent of degradation.

c. Feeding

When unmodified commercial brooder pens with external feeders are used, food spillage is likely to occur and some of the spilled food may fall into the feeders of lower pens in a stack. This could result in changes of test substance concentrations, and in some cases could significantly affect

the outcome of the test. A stacking arrangement of control pens on top with doses in lower pens being progressively higher toward the bottom of the stack has been used in the past in order to minimize the effects of spillage. Such an arrangement would provide the least amount of dosage change, but the resultant errors would tend to make a test substance appear somewhat safer than would occur without any spillage. In the majority of tests, the effects of spillage would probably be negligible. However, there are means to eliminate the effects of spillage.

The easiest method to avoid effects of food spillage is to not stack pens, however, this is usually impractical for reasons of space conservation. An effective method of minimizing spillage is to cover the feeding trays with hardware cloth or wire mesh of a size appropriate to the species being tested. Although this will reduce spillage, it will not eliminate it. Patuxent Wildlife Research Center has had good success by using 30 cm long commercial chick feeders and covering these with wire mesh (Hill, personal communication). These feeders are placed inside the pens and thus protected from spillage into other feeders. Another method for external feeders is to extend a solid piece of metal out from the pen (perhaps attached to the litter pan) to a point outside the feeder. Spillage may occur, but it will not fall into a lower tray. In addition to avoiding cross-contamination of concentration levels, the wire mesh over the feeder will help to provide accurate food consumption data.

Cross-contamination of two or more different test substances is avoided by requiring that only one substance

be used in any one stack of pen.

3. Facilities

Bobwhite dietary tests generally have been conducted in galvanized metal pens; mallard tests have been conducted either in galvanized metal pens or in wooden pens on straw-covered concrete slabs (Heath et al. 1972, Hill et al., 1975). The wooden pens were outdoors but were weatherproofed. The Agency's pesticide guidelines (US EPA 1978a) recommended commercial brooder units of wire mesh and galvanized sheeting, but no mention was made of outdoor pens. ASTM (1979a) stated that pens are best kept indoors where lighting, temperature, and other factors may be controlled. They recommended commercial brooder units of wire mesh and galvanized sheeting, but suggested that other materials would be adequate if they can be kept clean. The suggested construction materials were stainless steel, galvanized steel, and perfluorocarbon plastics. Materials that are toxic, capable of adsorbing test substances, or that alter or otherwise affect toxicity should not be used (ASTM 1979a).

For this test guideline, indoor pens were selected because they provide for control of more variables and contribute to the reproducibility of results. Outdoor pens, even when "weatherproofed", would result in test birds being exposed to different seasons, light regimes, temperature, disturbances, etc. In effect, they provide a substantially different environment. Therefore, the results of tests run in outdoor pens are not legitimately comparable with the results of tests run indoors. In addition, mallard tests conducted in outdoor pens limit the utility of comparisons

with bobwhite and other species that are normally tested indoors (Heath et al. 1972, Hill et al. 1975).

The effect of using various construction materials has not been documented. The original use of galvanized metal pens was based on what was available when the tests were developed. Galvanized metal is inexpensive and readily cleaned. Stainless steel is expensive, but is easier to clean than galvanized metal. There is some concern that studies with certain metals, such as zinc or nickel, could be affected by using galvanized metal or stainless steel, respectively (ASTM 1979b). Therefore, ASTM protocols (1979a, 1979b) include the possibility of using perfluorocarbon plastics (e.g., Teflon). Unfortunately, the perfluorocarbon plastics are not a sound structural material and have a tendency to warp and bend. Thus, cages constructed primarily of such materials are not particularly desirable. However, perfluorocarbon plastics may be used to coat other structural materials. Such pens should be examined regularly to ensure that the coating has maintained its integrity and has not exposed the underlying structural material. At the present time, it seems appropriate to allow the use of all three materials, although perfluorocarbon plastics are not recommended for structural parts.

The space requirements for birds are based on commercial pens that are commonly in use. Although no native birds are considered, ILAR (1978) specified 1451.7 square centimeters for individual pigeons and 232.3 square centimeters for Coturnix quail and for chickens under 500 grams weight. The sizes specified in the test rule are somewhat larger. The

larger size is expected to be less stressful because it allows some extra space appropriate to the interaction of test birds that are housed in groups. ILAR (1978) is also oriented towards laboratory animals that may be inbred to the point of tolerating more crowded conditions. Since the avian dietary test is designed to kill birds, extra space over that used for general laboratory animals will reduce the potential for stress from overcrowding to affect the results of the test.

Pens should be cleaned and sanitized between tests. Brushing and/or steam cleaning appear to be the most appropriate since they do not involve the use of chemicals that could affect subsequent tests. Detergents and bleach have been used by Denver Wildlife Research Center (Tucker, personal communication) and Patuxent Wildlife Research Center (Heinz, personal communication). The use of chemical disinfectants, such as quaternary ammonium compounds, should be avoided because of possibility that these compounds can leave toxic residues. However, the widely used cold sterilization method with ethylene oxide is acceptable, if needed for disease control. Pens should not be cleaned during a test in order to minimize disturbance to the test birds.

The choice of temperatures, humidity levels, and lighting regimes has been based upon historical precedents (Heath et al. 1972, Hill et al. 1975, US EPA 1978a, ASTM 1979a) and a lack of any proposed alternatives. A completely randomized cage arrangement is required. Pens are usually stacked on top of each other to enhance the utilization of space. A temperature gradient is strongly

recommended because the thermoregulatory ability of young birds varies among individuals and changes with age. The use of a gradient gives the young birds temperature options that are appropriate at varying times in their development of thermoregulatory ability.

The protocols developed by Patuxent Wildlife Research Center (Heath and Stickel 1965, Heath et al. 1972, Hill et al. 1975) did not specify any particular photoperiod. The Agency's Office of Pesticide Programs (US EPA 1975) stated that photoperiod may follow diurnal variation or be continuous for 24 hours per day. The next revision of the Pesticide guidelines (US EPA 1978a) stated a preference for continuous lighting but accepted a diurnal variation. ASTM (1979a) considered continuous lighting to be "probably optimum" but stated that a schedule of 14 hours light and 10 hours dark (14 L/10D) is acceptable. For this avian test guideline, a 14L/10D lighting schedule is recommended because it approximates natural lighting. A photoperiod that approximates the natural light regime is expected to be less stressful than exposure to constant lighting. Consideration was given to requiring, rather than recommending, a light-dark cycle. However, a lack of published data on the effects of continuous lighting versus a diurnal variation in young birds, along with a data base using continuous lighting (Hill et al. 1975) suggests that constant lighting should not be prohibited at this time. Research on this subject has been recommended in order to provide the Agency with additional information.

4. Environmental Conditions

a. Temperature (See Section II C.3)

b. Humidity (See Section II C.3)

c. Light (See Section II C.3)

D. Reporting Requirements

The information that is required to be reported in section II.B 6a, Statistical Analysis is essential to a proper evaluation of the test results. These required items are needed (1) to establish that the test was conducted according to specifications, (2) to evaluate those conditions and procedures that could affect the results of the test, and (3) to supply the Agency with sufficient information to conduct an independent analysis of statistics and conclusions. The location of the raw data storage will allow the Agency to find additional information that may have been left out of the report or that may be needed for enforcement purposes. The location is necessary because some chemical companies request the testing facility to keep these data, while other companies keep their own. The information is needed in a detailed manner because the avian dietary guideline contains few rigid requirements. Even when minimums or maximums are specified, it is important to know how much the test may have exceeded specifications, such as; if test birds were observed more frequently than required, if the number of test concentrations exceeded the five levels required, etc.

The information required in section II.B.6.b to be available, but not included in the test report, may be needed if there are serious concerns about the results or validity of the test. This information will not normally be

needed and therefore is not required routinely.

III. Economic Aspects

Three laboratories were surveyed to estimate costs for performing the test outlined in this guideline. The individual laboratories gave prices of $1850-2600, $850-1200, and $1500. The "best estimate" based upon the survey was $1510 for bobwhite and $1640 for mallards. A cost estimate also was made by separating the protocol into components and estimating the cost of each component, including direct labor cost, overhead cost, other direct cost, general and administrative cost, and fee. The best estimated final cost, based upon this calculation method, was $1973 for bobwhite and $2785 for mallards, with an estimated range of $\pm$ 50% of the best estimate. The calculated estimate is higher than the best estimate based on the survey. Differences in estimated prices or prices obtained from the different laboratories may have resulted from a number of factors, such as nature of the chemical, overhead rates, outside consultants, automation, marketing strategies, and other factors as outlined in a cost analysis report by Enviro Control (1980). The cost estimates were made assuming that the requirements of the Good Laboratory Practice Standards, as specified in Section (d) of the avian dietary toxicity guideline, are being satisfied.

In a cost analysis of subpart E pesticides guidelines, laboratories were surveyed in 1978 and in 1980 to determine the cost of testing (US EPA 1980a). The cited costs did not differentiate between species, however, the unit cost for an avian dietary LC_{50} test was $2000 in 1978 and $3600 in 1980.

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EG-16
August, 1982

BOBWHITE REPRODUCTION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

BOBWHITE REPRODUCTION TEST

(a) Purpose. This guideline is designed to develop data on the reproductive effects on the bobwhite of chemical substances and mixtures subject to chronic environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003 15 U.S.C. 2601 et. seq.). The Agency will use these and other data to assess the reproductive effects on birds that these chemicals may present to the environment.

(b) Definitions. (1) The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792--Good Laboratory Practice Standards apply here. In addition, the following definitions apply generally to this guideline:

(i) "Acclimation" Physiological and behavioral adaptation to environmental conditions (e.g., housing and diet) associated with the test procedure.

(ii) "Test substance" The specific form of a chemical or mixture of chemicals that is used to develop the data.

(iii) "Photoperiod" The light and dark periods in a 24 hour day. This is usually expressed in a form such as 17 hours light/ 7 hours dark or 17L/7D.

(iv) "Basal diet" The untreated form of the diet, such as the diet obtained from a commercial source.

(2) The definitions in this section refer specifically to the production and quality of eggs and the subsequent development of these eggs up to the point where young are 14 days old.

(i) "Eggs laid" This term refers to the total egg production during the test, which normally includes ten weeks of laying. Values are expressed as numbers of eggs per pen per season (or test).

(ii) "Eggs cracked" Eggs determined to have cracked shells when inspected with a candling lamp. Fine cracks cannot be detected without using a candling lamp and if undetected will bias data by adversely affecting embryo development. Values are expressed as a percentage of eggs laid by all hens during the test.

(iii) "Eggs set" All eggs placed under incubation, i.e., total eggs minus cracked eggs and those selected for analysis of eggshell thickness. The number of eggs set, itself, is an artificial number, but it is essential for the statistical analysis of other development parameters.

(iv) "Viable embryos (fertility)" Eggs in which fertilization has occurred and embryonic development has begun. This is determined by candling the eggs 11 days after incubation

has begun. It is difficult to distinguish between the absence of fertilization and early embryonic death. The distinction can be made by breaking out eggs that appear infertile and examining further. This distinction is especially important when a test compound induces early embryo mortality. Values are expressed as a percentage of eggs set.

(v) "Live 18-day embryos" Embryos that are developing normally after 18 days of incubation. This is determined by candling the eggs. Values are expressed as a percentage of viable embryos (fertile eggs).

(vi) "Hatchability" Embryos that mature, pip the shell, and liberate themselves from the eggs on day 23 or 24 of incubation. Values are expressed as percentage of viable embryos (fertile eggs).

(vii) "14-day old survivors" Birds that survive for two weeks following hatch. Values are expressed both as a percentage of hatched eggs and as the number per pen per season (test).

(viii) "Eggshell thickness" The thickness of the shell and the membrane of the egg at several points around the girth after the egg has been opened, washed out, and the shell and membrane dried for at least 48 hours at room temperature. Values are expressed as the average thickness of the several measured points in millimeters.

(c) Test procedures--(1) Summary of the test: (i) After birds have been obtained they should be observed for health and acclimated for at least two weeks.

(ii) Test birds should be randomly assigned to control and various treatment groups.

(iii) The test substance should be thoroughly and evenly mixed into the diet at concentrations specified in the test rule. All treatment levels should be analyzed for test substance concentrations at the beginning and midway through the test.

(iv) Birds should be weighed at the beginning of the test, at 14-day intervals until the onset of laying, and at termination of the test.

(v) Photoperiod should be carefully controlled on a short-day basis during the initial exposure phase, then increased to 16-17 hours to induce egg laying.

(vi) Birds should be observed regularly for abnormal behavior or mortality throughout the test.

(vii) Eggs should be removed daily and stored until there is a sufficient quantity for incubation. All eggs should be candled for cracks and cracked eggs removed. Once every two weeks, all eggs produced that day should be analyzed for eggshell thickness. Incubated eggs should be candled on day 11 and day 18. Hatching should be completed by day 24.

(viii) Hatchlings should be maintained until they are 14 days old. Abnormal behavior or death should be reported. Chicks should be weighed on day 14.

(ix) A statistical analysis should be performed, preferably by analysis of variance or regression analysis.

(x) The report should include all conditions, procedures, and results. Data should be sufficiently detailed for an independent statistical analysis.

(xi) All treated birds should be sacrificed and disposed of properly. Control birds may be kept as breeding stock, but should not be used in any other tests. Control offspring may be reared and used in another test as adults.

(2) [Reserved]

(3) [Reserved]

(4) Definitive test--(i) Test substance. (A) The concentrations of test substance in the diet will be specified in the test rule. Generally, three treatment groups and a control group will be used. The higher two treatment concentrations will be multiples (often 5x, 10x, or 20x) of the lowest treatment level. The highest treatment levels usually will be below lethal levels, unless predicted environmental exposure levels are high enough to approximate lethal concentrations.

(B) The material to be tested should be analytically pure

and the degree of purity should be reported along with the percentage of each impurity at levels specified in the test rule. If specifically required by a test rule for a particular substance or mixture, the technical grade should be tested. The test rule will specify the degree of purity or a range of compositions of the technical grade material.

(ii) Controls. A concurrent control is required during every test. The control birds should be from the same hatch as the test groups. Control and test birds should be kept under the same experimental conditions. The test procedures should be the same for control and treated birds, except that no test substance should be added to the diets of control birds. If a carrier or diluent is used in preparation of the test diets, the same carrier should be added to the diets of control birds in the highest concentration used for test diets. The use of shared controls is acceptable for concurrent tests as long as the same carrier is used for all the tests.

(iii) Test groups and numbers of birds. (A) Each of the three treatment groups and the control group should consist of a minimum of 12 replicate pens. Each pen should contain one male and one female, or alternatively one male and two females. The use of 20 replicate pens in the control group may yield a test with greater statistical power. Either arrangement is acceptable

productivity reaches the definitive values given in paragraph (c)(4)(xii) of this section. Because the behavioral interactions of birds in the two arrangements is likely to be different, testing facilities using an arrangement with which they are not familiar are advised to experiment first without test substances in order to determine the feasibility of obtaining acceptable productivity levels.

(B) All control and treatment birds should be randomly distributed to pens from the same population.

(iv) Duration of test. (A) The test consists of three phases following acclimation to test facilities. The initial phase begins with exposure of treatment groups to diets containing the test substance and is typically six to eight weeks long. After the initial phase, the photoperiod is manipulated according to paragraph (c)(4)(v) of this section to bring the hens into laying condition. This second phase ends with the onset of egg-laying and is typically two to four weeks long. The final phase begins with the onset of laying and lasts for at least eight weeks, preferably ten weeks. A withdrawal study period may be added to the test phase if reduced reproduction is observed. The withdrawal period, if used, need not exceed three weeks.

(B) Exposure of adult birds to the test substance should be continuous throughout the test. Unless otherwise specified in the test rule, test birds should be exposed for at least ten weeks prior to the onset of egg laying.

(v) Preparation for reproduction (photoperiod). (A) Lighting regimes (photoperiod) are critical to successful reproduction. Various photoperiod regimes have been demonstrated to give acceptable results. Any photoperiod regime that results in productivity that meets the definitive values given in paragraph (c)(4)(xii) of this section is acceptable as long as birds are exposed to treated diets a minimum of ten weeks prior to the onset of laying. Regardless of the methods selected, lighting should be controlled carefully. It is important during the initial phase to not interrupt the dark period unless absolutely necessary.

(B) A suggested photoperiod regime would consist of maintaining birds under a photoperiod for seven or eight hours of light during the initial phase. At the end of the initial phase, the photoperiod may be increased to 16-17 hours of light per day. The photoperiod may be maintained at this level for the remainder of the study, although an increase each week of 15 minutes per day is acceptable.

(vi) Observations of record on adult birds. (A) Body weights should be recorded for each adult bird at the beginning of the treatment period, at 14-day intervals until the onset of egg laying, and at termination of treatment. Recording of body weights during egg laying is discouraged because of possible adverse effects on egg production. Food consumption should be measured and recorded by pen as often as body weights are measured prior to the onset of laying and at least bi-weekly throughout the rest of the study.

(B) Observations on adult birds should be made at least once a day. Any mortality or other signs of toxicity should be described and recorded by date or day of test. Gross pathological examinations should be conducted on all birds that die during the test period, and for all survivors at the end of the test. Analysis of two or more tissues (e.g., muscle, fat) for test substance residues is encouraged, but not required (unless specified in the Test Rule).

(vii) Egg collection, storage, and incubation. All eggs should be collected daily, marked according to the pen from which collected, and should be stored at 16°C and 55-80 percent relative humidity. Storage in plastic bags may improve uniformity of hatching. Stored eggs should be turned daily. At weekly or bi-weekly intervals, eggs should removed from storage

and be candled to detect eggshell cracks. Except for eggs with cracked shells and those eggs removed for eggshell thickness measurements, all eggs should be set after candling for incubation in a commercial incubator. If incubators are not equipped to automatically turn eggs, they should be turned daily by hand. During the incubation period, eggs should be maintained at 37.5°C and approximately 70 percent relative humidity. Eggs should be candled again on day 11 of incubation to determine fertility and early death of embryos. A final candling should be done on day 18 to measure embryo survival. On day 21, eggs should be removed to a separate incubator or hatcher. Hatching will normally be complete by the end of day 24.

(viii) Chick maintenance. By day 24 of incubation, the hatched bobwhite chicks should be removed from the hatcher or incubator. Chicks should be either housed according to the appropriate parental pen group or individually marked (such as by leg bands) as to parental group and housed together. Chicks should be maintained in commercial brooder pens or pens of similar construction. Pens should be constructed of galvanized metal or stainless steel. Temperature in the pens should be controlled, preferably by a thermostatically control device. A temperature gradient in the pen from approximately 35°C to approximately 22°C will allow young birds to seek a proper

temperature. Temperature requirements for young birds typically decline over this range from birth through the first several weeks of life. Chicks should be provided a standard commercial game bird starter ration, or its nutritional equivalent. No test substance may be added to the diet of chicks. Chicks should be maintained until they are 14 days old.

(ix) Observations of record on chicks. The hatchability, percentage of normal hatchlings, percentage of 14-day old survivors, and number of 14-day old survivors per hen should be recorded and reported. Chicks should be observed daily from hatching until they are 14 days old. Mortality, signs of toxicity, and other clinical abnormalities should be recorded at least cumulatively through day 5 and recorded by age from days 5 through 14. Average body weights should be recorded for chicks at day 14.

(x) Eggshell thickness. Once every two weeks all eggs newly laid that day should be removed and measured for eggshell thickness. Eggs should be opened at the girth (the widest portion), the contents washed out (or used or saved for egg residue analysis), and the shells air dried for at least 48 hours. The thickness of the shell plus the dried membrane should be measured at a minimum of 3 points around the girth using a micrometer calibrated at least to 0.01 mm units.

(xi) Typical observed values The values reported here represent those observed from a few testing facilities under their conditions. These values are not necessarily representative of those from all facilities, however, if a reproduction test does not meet or at least approach these values for control birds, then there is likely to be a problem with test procedures or conditions that should be investigated and corrected.

(A) Eggs laid. Normal values for bobwhite - 28 to 38 eggs per hen per season.

(B) Eggs cracked. Normal values for bobwhite - 0.6 percent to 2.0 percent of eggs laid.

(C) Viable embryos (fertility). Normal fertility values for bobwhite - 75 percent to 90 percent of eggs set.

(D) Live 18-day embryos. Normal values for bobwhite - 97 to 99 percent of viable embryos.

(E) Hatchability. Normal values for bobwhite - 50 percent to 90 percent of viable embryos (fertile eggs).

(F) 14-day-old survivors. Normal values for bobwhite - 75 percent to 90 percent of eggs hatched.

(G) Eggshell thickness. Normal average values for bobwhite - 0.19 mm to 0.24 mm.

(xii) Definitive test criteria. (A) A test is unacceptable if bobwhite chick productivity in control groups does not average twelve 14-day old survivors per pen over a ten week period.

(B) A test is unacceptable if the average eggshell thickness in control groups is less than 0.19 mm.

(C) A test is unacceptable if more than 10% of the adult control birds die during the test.

(5) [Reserved]

(6) Analytical measurements--(i) Statistical analysis. Experimental groups should be individually compared to the control group by analysis of variance. Other accepted statistical methods may be used as long as they are documented. In particular, regression analysis is highly desirable if the data and number of dose levels allow the use of this technique. Sample units are the individual pens within each treatment level or control. Analysis should include:

(A) Body weight of adults.

(B) Food consumption of adults.

(C) Percentage of hens laying eggs. This should always be determined when pens contain a single pair; if feasible, it should be determined when pens contain groups.

(D) Number of eggs laid per pen.

(E) Percentage of cracked eggs.

(F) Percent viable embryos of eggs set.

(G) Percent live 18-day embryos of viable embryos.

(H) Percent hatching of viable embryos.

(I) Percentage of hatchlings that are normal.

(J) Percent 14-day-old survivors of normal hatchlings.

(K) Number of 14-day-old survivors per hen.

(L) Body weights of 14-day-old survivors.

(M) Eggshell thickness.

(ii) Test substance concentrations. (A) Samples of treated diets should be analyzed to confirm proper dietary concentrations of the test substance. If samples cannot be analyzed immediately, they should be stored appropriately (e.g., frozen at a temperature of -15°C or lower) until analysis can be performed. Analyses should be conducted on all test substance concentrations at the beginning of the test period and again 10 to 12 weeks later. If not otherwise available, data should be generated to indicate whether or not the test substance degrades or volatilizes. If the test substance is known or found to be volatile or labile to the extent that 25 percent or more loss occurs within one week, then test substance diets should be prepared (freshly or from frozen concentrate) at a frequency that will prevent more than 25 percent% loss of test substance.

(B) The assay method used to determine actual concentrations should be reported according to paragraph (e)(1)(vi) of this section.

(iii) Analysis of Basal Diet. A nutrient analysis of the basal diet should be included with the test report. For commercially prepared basal diets, the list of ingredients supplied by the manufacturer is normally sufficient if it is detailed. The composition of any vitamin or other supplements should also be reported.

(d) Test conditions--(1) Test species--(i) Selection. (A) Bobwhite, Colinus virginianus (L.), is the test species. Test birds should be pen-reared. They may be reared in the laboratory or purchased from commercial breeders. Rearing stock and/or test birds should be obtained only from sources that have met the requirements for "U.S. Pullorum-Typhoid Clean" classification. Birds should be obtained only from sources whose colonies have known breeding histories. If possible, a history of rearing practices for test birds should be obtained and made available upon request. This history should include lighting practices during rearing, disease record, drug and any other medication administered, and exact age. Test birds should be phenotypically indistinguishable (except for size) from wild stock. Conscientious breeders of such birds will periodically

outbreed their flocks with genetically wild stock in order to maintain a genetic composition that approximates the heterogeneity of naturally occurring birds.

(B) All control and experimental birds used in a test should be from the same source and strain. If shipped, all birds should be examined following shipment for possible physical injury that may have occurred in transit. All birds should have a health observation period of at least two weeks prior to selection for treatment. Birds should be in apparent good health. Deformed, abnormal, sick, or injured birds should not be used. A population of birds should not be used if more than 3 percent of either sex die during the health observation period. Birds should not have been selected in any way for resistance to toxic substances. Birds should not have been used in a previous test, either in a control or treatment group. Offspring of birds used in a treatment group in a previous test should not be used, but offspring of birds used as a control in a previous test are acceptable.

(C) Test birds should be approaching their first breeding season and should be at least seven months old. All test birds should be the same age within one month. The age of test birds should be reported.

(D) Bobwhite should be acclimated to test facilities and untreated basal diet for at least two weeks. Acclimation may be in the actual pens used in the test or in identical pens. The acclimation period may coincide with the health observation period. Birds should be randomly assigned to treatment and control pens. However, when birds in a pen are incompatible, they may be rearranged within a control or treatment group at any time prior to initiating treatment.

(E) During holding, acclimation, and testing, birds should be shielded from excessive noise, activity, or other disturbance. Birds should be handled only as much as is necessary to conform to test procedures.

(ii) Diet--(A) Adult birds. (1) A standard commercial game bird breeder ration, or its nutritional equivalent, should be used for diet preparation. This ration or basal diet should be used for both control and treatment birds and should be constant throughout the duration of the study. Antibiotics or other medication should not be used in the diet or water of breeding birds. It may not be possible to obtain food that is completely free of pesticides, heavy metals, and other contaminants. However, diets should be analyzed periodically for these substances and should be selected to be as free from contaminants as possible. A nutrient analysis (quantitative list

of ingredients) of the diet should be included with the test report.

(2) The test substance should be mixed into the diet in a manner that will ensure even distribution of the test substance throughout the diet. If possible, the test substance should be added to the diet without the use of a carrier or diluent. If a diluent is needed, the preferred diluent is distilled water; but water should not be used for test substances known to hydrolyze readily. When a test substance is not water soluble, it may be dissolved in a reagent grade evaporative diluent (e.g., acetone, methylene chloride) and then mixed with the test diet. The solvent should be completely evaporated prior to feeding. Other acceptable diluents may be used, if necessary, and include table grade corn oil, propylene glycol, and gum arabic (acacia). If a diluent is used, it should comprise no more than 2 percent by weight of the treated diet, and an equivalent amount of diluent should be added to control diets.

(3) Diets may be mixed by commercial or mechanical food mixers. Other means are acceptable as long as they result in even distribution of the test substance throughout the diet. Screening of the basal diet before mixing is suggested to remove large particles. For many test substances, it is recommended that diets be mixed under a hood. Frequently, the test substance

is added to an aliquot of the basal diet to form a premix concentrate. The premix concentrate should be stored so as to maintain the chemical concentration. For final preparation of test diets, the premix is mixed with additional basal diet to form the proper concentrations. The frequency with which final treated diets are prepared will depend upon the stability and other characteristics of the test substance. Unless otherwise specified in the test rule or determined by degradation or volatility studies, it is recommended that final diets be prepared weekly, either fresh or from a concentrate. For volatile or labile test substances, test diets should be mixed frequently enough so that the concentrations are not reduced from initial concentrations by more than 25 percent. Analysis of diets for test substance concentration is required as specified in paragraph (c)(6)(ii).

(4) Clean water should be available ad libitum. Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water should be changed daily or more often.

(B) Young birds. Young birds produced during the test should be fed a commercial game bird starter ration, or its nutritional equivalent. No test substance should be added to the diets of young birds. No antibiotics or medication may be used in the diet. Bacitracin, or one of its forms, may be added to

the drinking water of young birds, if necessary.

(2) Facilities. (i) Bobwhite should be housed in breeding pens or cages of adequate size conforming to good husbandry practices. Space requirements for bobwhite have not been well defined, but it is recommended that there be at least 5000 square centimeters (approximately 2.7 square feet) of floor space per bird. Documentation that reproductive parameters and health of birds are not adversely affected should be provided for cages much smaller than this area. The preferred construction materials are stainless steel, galvanized sheeting, and wire mesh. For enclosed cages, floors and external walls may be wire mesh; ceilings and common walls should be solid sheeting. Wire mesh for floors should be fine enough so as to not interfere with normal movement of bobwhite. Open-topped pens may be constructed of the same materials for the side walls with open tops and wire mesh or concrete floors. Concrete floors should be covered witn litter such as straw, wood shavings, or sawdust. Other construction materials, except wood, are acceptable if they can be kept clean. Wood may be used as vertical framing posts for the support of wire mesh or for horizontal framing along the top of the pen. Wood should not be used for floors or lower sides of pens unless it has been coated with a non-adsorbent material such as perfluorocarbon plastic (e.g., Teflon), or unless the wood is

replaced between tests.

(ii) Pens should be disassembled (if feasible) and should be cleaned thoroughly between tests. Steam cleaning of enclosed cages is recommended. Enclosed cages may be brushed thoroughly, as an alternative method. For open-topped pens, the sides and vertical supports should be thoroughly brushed. Any used floor litter should be discarded. The floor composition will dictate methods used to clean the floor. If litter is used on the floor, it should be fresh and clean when birds are placed in the pen. The use of detergents or bleach is acceptable, but other chemical disinfectants (such as quaternary ammonium compounds) should not be used. When necessary to control disease vectors, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended.

(iii) Pens should be kept indoors in order to better control lighting, temperature, humidity, and other factors. Outdoor pens may be used only during the normal breeding season. The photoperiod should be carefully controlled, preferably by automatic timers. A 15-30 minute transition period is desirable. The photoperiod regime is described under test procedures under paragraph (c)(4)(v). Lights should emit a spectrum simulating that of daylight. The use of shorter wave-

length "cool-white" fluorescent lights that do not emit the daylight spectrum should be avoided. Illumination intensity should be about 6 foot-candles at the level of the birds.

(iv) Temperature and humidity should be controlled during the study. Recommended levels are 21°C and 55 percent relative humidity. Temperature should be recorded at least weekly at the same time of day and should be reported. For tests conducted without temperature control, temperature minimums and maximums should be recorded daily. Continuous temperature monitoring is desirable. Temperature recordings should be made at a level of 2.5-4 cm above the floor of the cage. Recording of approximate humidity levels is also desirable. Good ventilation should be maintained. Suggested ventilation rates are 4 changes per hour in winter and 15 changes per hour in summer.

(v) If facilities are being used for the first time, it may be desirable to allow birds to breed in the facility prior to testing in order to ensure that controls will have acceptable productivity according to the requirements given in paragraphs (c)(4)(xi) and (xii).

(3) [Reserved]

(e) Reporting. (1) The test report should include the following information:

(i) Name of test, sponsor, test laboratory and location,

principal investigator(s), and actual dates of beginning and end of test.

(ii) Name of species tested (including scientific name), age of birds (in months) at the beginning of the test, source of birds, and body weights for adult birds throughout the test.

(iii) Description of housing conditions, including type, size, and material of pen, temperature, humidity, photoperiod and lighting intensity, and any changes during the test.

(iv) Detailed description of the basal diet, including source, composition, diluents (if used), and supplements (if used). A nutrient analysis of the basal diet should be included.

(v) Detailed description of the test substance including its chemical name(s), source, lot number, composition (identity of major ingredients and impurities), and known physical and chemical properties pertinent to the test (e.g., solubility, volatility, degradation rate, etc.).

(vi) The number of concentrations used, nominal and measured concentrations of test substance in each level, assay method used to determine actual concentrations, storage conditions and stability of treated diets, number of birds per pen and number of replicate pens per concentration and for controls.

(vii) Acclimation procedures and methods of assigning birds to test pens, including method of randomization, and any

rearrangement due to incompatibility.

(viii) Frequency, duration, and methods of observation.

(ix) Description of any signs of intoxication, including time of onset, duration, severity (including death), and numbers affected, including accidental deaths or injuries.

(x) Food consumption per pen and any observations of repellancy or food palatability.

(xi) Method of marking all birds and eggs.

(xii) Details of autopsies.

(xiii) Egg and hatching data in summary and by pen per week in sufficient detail to allow an independent statistical analysis. Data should be presented for all of the parameters listed in paragraph (c)(6)(i). The number of eggs set should also be reported.

(xiv) Egg storage, incubation, and hatching temperatures, relative humidities, and turning frequencies.

(xv) Observations of health and weights of young at 14 days of age.

(xvi) Location of all raw data storage.

(xvii) Methods of statistical analysis and interpretation of results.

(xviii) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(2) In addition, the following information should be available upon request:

(i) A general description of the support facilities.

(ii) A description of the Quality Control/Quality Assurance program, including the Average Quality Level for the program element performing the test, procedures used, and documentations that these levels have been achieved.

(iii) The names, qualifications, and experience of personnel working in the program element performing the test, including the study director, principal investigator, quality assurance officer, as well as other personnel involved in the study.

(iv) Standard operating procedures for all phases of the test and equipment involved in the test.

(v) Sources of all supplies and equipment involved in the test.

(vi) Diagram of the test layout.

(vii) Originals or exact copies of all raw data generated in performing the test.

(viii) A detailed description, with references, of all statistical methods.

ES-13
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

BOBWHITE REPRODUCTION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TEST SUPPORT DOCUMENT FOR BOBWHITE REPRODUCTION TEST

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-16 which uses Bobwhite to evaluate the effect of chemical substances on reproduction. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescribed in the Test Guideline. Technical issues and practical considerations are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. General

The earliest investigations of the effects of chemicals on reproduction of native birds were in the 1950s (DeWitt 1956, Genelly and Rudd 1956). Chemicals were administered in the diet, but procedures varied. Laboratory investigation of reproductive effects of pesticides continued at Patuxent Wildlife Research Center, but methods were not reported well (DeWitt et al. 1962, DeWitt et al. 1963). In 1964, a very brief protocol for reproduction studies was developed at that center (Stickel and Heath 1965). This protocol outlined the egg parameters to be studied. Ratcliffe (1967), in a classic paper, correlated the decline of certain avian populations with thin eggshells that apparently had resulted primarily from exposure to DDT and DDE. Heath et al. (1969) presented the first clearcut experimental data showing that DDE caused thin eggshells in

mallards. The protocol used by Heath et al. (1969), although not fully detailed, formed the basis for most future avian toxicological reproduction studies. In 1968, USDA developed a guideline for evaluating reproductive effects of toxic chemicals to birds (US EPA 1975). This guideline apparently was developed in conjunction with Patuxent personnel as it bears a great similarity to methods used at Patuxent, but was available prior to the publication of Heath et al. (1969). The Agency (US EPA 1975) developed composite protocols for reproduction tests from the limited published information and unpublished information, especially the USDA protocol which was presented as an exhibit. No complete, suitable protocol for bobwhite was available from the published literature at that time (US EPA 1975), although Heath et al. (1972b) and Heath and Spann (1973) had published the results of bobwhite reproduction studies. The Agency's pesticide guidelines were revised (US EPA 1978a) but the basic method of the earlier guidelines for reproduction tests was retained. There have been very few bobwhite reproduction tests published even in recent years, although a number of tests have been conducted, classified as confidential, and submitted to the Agency to support pesticide registration. ASTM (1979a) has prepared a draft avian reproduction method. Although this ASTM protocol is similar to the Office of Pesticides Programs protocol (US EPA 1978a), it is designed for a variety of species. Thus, the history of avian reproduction test methodology is basically a history of Patuxent and EPA methods. This guideline continues the trend because these methods appear to be the most appropriate for developing

data needed to make regulatory decisions and because no other methods have become accepted widely.

Even though the basic method is similar for most investigators, there have been a few points of difference or controversy. Yet there are very few data to address these differences and little ongoing research that might resolve conflicts. To promote uniformity and comparability of tests, some recommendations and requirements are standardized in this test guideline. Where such recommendations and requirements are controversial and are not sufficiently addressed by published data, it is hoped that research will be stimulated to resolve questions. If feasible, conditions and procedures that approximate natural conditions have been selected in preference to other options.

The use of avian reproduction tests in the assessment of chemical impacts on the environment is based on several factors. First, birds are an obvious and important component of the environment. Congress has indicated repeatedly that birds are worthy of protection by passing such laws as the Lacey Act of 1900, Migratory Bird Treaty Act of 1918, Migratory Bird Conservation Act of 1929, Pittman-Robertson Act of 1937, Fish and Wildlife Act of 1956, Endangered Species Act of 1973, and others. The United States also has entered into treaties with Great Britain and Canada (1916), Mexico (1937), Japan (1974), and Russia (1976) for the protection of migratory birds. The people of the United States also have indicated a desire to protect birds through their support of the Audubon Society, Nature Conservancy, and other environmental groups.

Sportsmen's organizations support protection of birds, although their interests often focus heavily on game birds.

Second, birds have a definite economic importance. Federal and State Agencies spend large sums for the preservation and propagation of birds. Hunters and birdwatchers also spend substantial sums in pursuit of their pastimes. Less measurable, but of definite importance, is the substantial role of birds in insect control.

Birds have an important ecological role. Insectivorous birds consume huge quantities of insects and other invertebrates, many of which are considered pests. Small mammals and other vertebrates or plants are consumed by various birds, sometimes to the extent that birds have an important effect on populations. In turn, birds are consumed by birds of prey, mammals, and other vertebrates. Excretory products of birds provide nutrients for plankton and other microorganisms that in turn are food for larger organisms. Birds are important in pollination of some plants and in dispersal of others. Because of their mobility, the effects of birds are not restricted to specific locations.

Finally, birds are among the more sensitive terrestrial vertebrates. Because of their high metabolic rate, high body temperature, and the demands of flight, they require more energy relative to their size than most other animals. The energy requirements lead to greater food intake and thus to greater toxicant intake when a toxicant is in or on their food. There are abundant data showing that some birds, particularly raptors, pelicans, and waterfowl, are very sensitive in their reproductive responses to toxic chemicals (e.g., Ratcliffe 1967, Anderson

and Hickey 1972) and that some species have suffered from drastic population reductions apparently as a result. Avian reproduction is unique, and no surrogate is adequate to predict effects on eggshells, the primary mode of impairment for many chemicals such as DDE (Heath et al. 1969).

Avian reproduction tests are extremely valuable in assessing the potential population effects of exposure of birds to toxic chemicals. The route of intake simulates natural exposure to chemicals on or in the food. Most physiological effects can be assessed under laboratory conditions, although many behavioral effects such as nest desertion are difficult to study in the laboratory. A positive finding of impairment in the laboratory is highly predictive (qualitatively) in the field when exposure is the same. However, negative findings in the laboratory may not preclude adverse effects under field conditions. Thus, some extrapolation may be made from laboratory to field, but quantitative extrapolation is risky.

1. Issues

The avian reproduction test guideline and support document contain some controversial points. Data are insufficient or absent to support either side of most points. For other points, there may be data supporting each side. A number of controversial points have been selected as potential research projects. Issues are merely identified below and are discussed in the appropriate sections of this document.

- Is productivity from tests run out of normal season sufficient to evaluate the potential for reproductive impairment?

- Should methods involving natural incubation of eggs be incorporated into tests?
- Should the current use of first year birds only be continued or should proven breeders be used? Would either age produce results suitable for comparison?
- What carriers, if any, should be used or allowed for incorporation of the test substance into the diet?
- Are commercial foods adequate, or is there too much variation and/or contamination of commercial foods?
- How often should treated diets be mixed? Is there an advantage in mixing diets with decreasing concentrations to simulate natural degradation of test substances?
- What is the optimum number of birds to be tested in order to attain statistically valid results and still be cost-effective? Can tests using pairs only of birds in pens be successfully conducted by a variety of testing facilities?
- Can more useful results be obtained by testing enough dose levels to use linear regression analysis?
- Should outdoor tests be allowed?
- Should medication be allowed as is needed to treat individual sick birds?
- Are the typical values for productivity and other egg parameters realistic for a wide diversity of testing facilities, or are they really only valid for a few?

B. Test Procedures

1. Test Substance Concentrations

Test substance concentrations in the diet will be specified in the test rule. These concentrations will be based upon the properties of the test substance, the lethal and no-effect levels, if known, or the amount of test substance known or likely to be found in the environment. Three concentration levels are specified because, for many test substances, three levels will allow for a dose-response regression analysis from which a no- effect level can be calculated. (See section 2.1.8 on statistics for further discussion).

2. Controls

Concurrent controls are required for every test to assure that any observed effects are a result of ingestion of the test substance and not of other factors. Such other factors may include environmental factors such as temperature or lighting, vapors, sensitive or stressed test birds, etc. If a diluent is used in mixing the diet, this diluent also is used in the untreated diets in the same concentration as it occurs in treated diets. In effect, this results in a diluent, but no completely negative, control. Diluent choices are based upon their lack of toxicity (e.g., water, completely evaporated acetone) and it is not considered necessary, therefore, to have an additional negative control when a diluent control is used.

3. Test groups and numbers of animals

A minimum of 12 replicate pens with one cock and two hens or 20 replicate pens with one cock and one hen is required for each test concentration. The number of

replicates is needed to achieve a test with statistical significance. There is enough variation in productivity of bobwhite hens that fewer replicates would be insensitive to all but the most severe effects.

The most recommended arrangement is for use of group pens containing two females each. This is based upon the rather agressive behavior of courting males that could result in severe stress to the female, if only one hen were present. In reproduction tests different from this guideline and other published protocols, Kendall et al. (1978) used pairs of bobwhite. Currently, K. Stromberg (personal communication) has been working on reproduction tests with pairs of bobwhite at Patuxent Wildlife Center (USDI). Data from Stromberg's work are still being analyzed, but it is expected that methods for keeping bobwhite pairs successfully will result from that research. The alternative arrangement of 20 bobwhite pairs per level is suggested to accommodate those test facilities that believe they will have success or that wish to contribute to the development of methods. A warning is included in the guideline so that relatively inexperienced investigators should not expect unqualified success. The additional pens in pair testing will strengthen notably the statistical analysis of the test. This alternative is included because it is statistically stronger than the more familiar method of group testing.

4. Duration of Test

The avian reproduction test lasts approximately 22 weeks. The initial part of the test is an exposure phase where birds are receiving treated diet. Exposure to treated

diets begins with this phase and allows for the test substance to act upon the reproductive mechanisms of the body, and also for birds to accumulate residues of lipophilic and other test substances. The development of reproductive capacity actually begins months before egg laying starts (Kirkpatrick 1959). Therefore, exposure to the test chemical should be well in advance of egg laying, if the test is to investigate reproductive effects in general.

The second phase, following lengthening of the photoperiod, directly brings the birds into readiness for egg laying. The duration of this phase is dependent upon the response of the test birds.

The third phase is the egg-laying portion of the test. This is to be a minimum of eight weeks. The duration of this phase is based upon two main factors. First, it is important to determine if egg laying is within normal levels or if it declines, or otherwise varies, over a period of time. This yields information on speed of action and has a role in using the test for assessment purposes. Second, the eight week period is needed to provide sufficient data for a strong statistical analysis.

A withdrawal period is optional when impairment has been detected. This period may provide data on recovery of reproductive capacity that could be useful for assessment purposes.

5. Preparation for reproduction (photoperiod)

Because photoperiod is critical to reproduction, it should be controlled in indoor tests. Under natural conditions, photoperiod is lengthening gradually just prior

to the reproductive season. The earlier bobwhite reproduction tests were conducted outdoors (Stickel and Heath 1965, Heath et al. 1972b, Heath and Spann 1973), and therefore, used natural photoperiod. More recently, bobwhite tests involving reproduction have been conducted indoors (Fergin and Schafer 1977, Kendall et al. 1978) and egg laying has been induced by increasing photoperiod to 16 or 17 hours of light per day. The ASTM (1979a) and US EPA (1978a) protocols both recommended seven hours of light in the first phase and 16-17 hours light in the second phase. Without giving supporting data, both of these protocols stated that the regime is for maximum egg production, and both specify at least an option of gradually increasing the length after egg laying has started. The photoperiod in this guideline is based on the above data and a lack of any suggested alternatives.

The dark period in the photoperiod should not be interrupted, even briefly, except as absolutely necessary. Kirkpatrick (1955) found that even as little as 15 minutes interruption on a short day/long night regime caused an increase in gonadal development, and all birds exposed to a 60 minute interruption became fully active sexually. It is highly probable, on the basis of Kirkpatrick's (1955, 1959) data, that it is the length of the dark period rather than the light period that controls reproductive preparation.

Kirkpatrick (1955) also tested light intensity as a factor in bobwhite reproduction. Responses were very similar at intensities of 0.1 to 100 foot candles on a 17 hour light photo- period. With shorter days, the 0.1 foot candle intensity did not achieve the same results as 1,10,

and 100 foot candles. These data support the approximate 6 foot candle intensity suggested by ASTM (1979a) and US EPA (1978a).

6. Observations and Measurements

a. Adult Birds

Observations of food consumption, body weights, and signs of toxicity are required. Body weights are required three times during the test. More frequent body weights might be informative, but the stress of handling may offset the collection of data, particularly for laying hens. Food consumption is to be estimated at frequent intervals. This will provide data both on test substance ingestion and on energy intake for test birds. The latter data will be particularly helpful in the absence of frequent weighings.

Clinical signs of toxicity contribute substantially to the analysis of the data, in addition to providing information on the mode and speed of action. If dose levels are finely tuned, there should be minimal observed acute toxicity. However, without adequate preliminary data, one or more of the test levels may cause lethal or notable sublethal effects. Such effects may affect the results of the test without acting on the specific reproductive parameters being investigated. For example, a severely stressed bird may not be able to mobilize internal resources to produce eggs. Signs of toxicity will give valuable information in evaluating the results of the test.

b. Chicks

The incubation period for bobwhite eggs has been reported to be 559± 7 hours (23.3 days) (Wetherbee and Wetherbee, as cited in ILAR 1977). Some variation may occur

as a result of temperature or humidity conditions (ILAR 1977). Bobwhite eggs have been incubated for 24 days (Fergin and Schafer 1977) or 25 days (Heath and Spann 1973). US EPA (1978a) stated that eggs should be removed from hatchers on day 24. The meager data available suggest that 24 days is sufficient and this incubation length has been selected for this guideline.

Because the pen is the basic unit for statistical analysis, it is necessary that the eggs and hatchlings be identified as to pen of origin. This can be done either by housing all chicks from one pen together, or by individually marking each egg and bird.

Environmental conditions for young chicks are based upon historical precedents (Heath et al. 1972, Hill et al. 1975) and other current methods for maintaining young chicks (ASTM 1979b, US EPA 1978b). Parameters given in these references include temperature of 35°C with a lower temperature outside the cage to provide a gradient, galvanized brooder cages maintained indoors, and a commercial game bird starter ration. These conditions have been included in this guideline.

Observations on chicks are necessary to determine if and when toxicity might be expressed in the offspring. Although most test substances exert their action prior to hatching, some may affect growth, development, or survival of chicks.

c. Eggshell Thickness

The classic example of reproductive impairment in birds is the eggshell thinning effect of DDE, a metabolite or degradate of DDT (Tucker and Leitzke 1979). It is important to measure eggshell thickness because as little as 11%

thinning may have an effect on avian populations (Tucker and Leitzke 1979). Techniques given in this guideline are widely used (Heath and Spann 1973, ASTM 1979a, US EPA 1978a), although many papers do not give a full description of the process. It is particularly important for several measurements to be made around the girth of bobwhite eggs, because these eggs may have small calcium deposits that could affect results if only one or two measurements are taken.

d. Typical observed values

The typical observed values presented in this guideline have been taken from the Agency's pesticide guidelines (US EPA 1975, 1978a). A number of avian reproduction tests have been submitted to the Agency for the purpose of pesticide registration and have been classified as confidential. Most of the tests that have been conducted during the normal reproductive season have achieved for control birds the typical values as presented in the test guideline. Heath and Spann (1973) and Fergin and Schafer (1977) achieved these values in control birds insofar as could be determined from their data. Heath et al. (1972b) did not quite reach the egg production values in an eight week season for a test conducted outdoors with three hens and 2 cocks per pen. They did achieve all other normal values except that for cracked eggs. Ten week indoor tests according to this guideline would likely have egg production values that correlated with these normal values.

The values presented in the guideline have been identified as an issue. It is known that some testing facilities routinely

meet these norms. However, it is not known if the values are realistic when many testing facilities are considered.

7. Egg Collection, Storage, and Incubation

Egg storage prior to setting for incubation has been reported for bobwhite in only a few papers and has ranged from 16°C and 55% humidity (Heath et al. 1972b) to 19°C and 70° humidity (Kendall et al. 1978). Stromberg (personal communication) stated that he stored eggs at 16° and as high a humidity as could be obtained from evaporating water; this was up to 80%, but at least 50% relative humidity. He also stated that turning the eggs daily was important to keep part of the shell and egg from excessive drying. US EPA (1978a) recommends 16°C and 65% relative humidity. This guideline specifies 16°C, but only a range of relative humidity between 55% and 80%, since many testing facilities do not have the means to control storage humidity and since this range has produced good viability.

No papers were found that reported incubation temperatures and humidities for bobwhite. Neither ASTM (1979a) nor US EPA (1978a) specify incubation temperatures although the latter does recommend 39°C and 70% relative humidity during the hatching phase from day 21 to day 24. Stromberg (personal communication) stated that 99.75°F (37.6°C) and 50-70% relative humidity are standard commercial poultry incubation temperatures and that all testing facilities with which he is acquainted use commercial poultry incubators. Mallards have been incubated at temperatures of 37.4-37.5°C and relative humidities of 62-80% (Heinz 1976a, Heinz 1976b, Greenwood 1975, Holmes et al. 1978). These same parameters have been specified in

this bobwhite test guideline.

Candling procedures are a standard practice for determining eggshell cracks, fertility, and embryo survival. These procedures have been used in most bobwhite tests, but timing has been inadequately reported. US EPA (1978a) specifies, for bobwhite, candling on day 0 for cracks, on day 11 for fertility and on day 18 for embryo survival. This timing has been adopted in this guideline because it will permit comparisons of test data with existing studies in Agency files. At least one major avian testing laboratory protocol uses the same timing.

US EPA (1978a) recommended moving eggs to a separate hatcher or incubator for hatching, but gave no reasons. None of the available published papers mentioned this procedure. However, Stromberg (personal communication) stated that this procedure is highly recommended as a means to minimize disease vectors. This procedure has been recommended in the guideline.

This test guideline does not consider the effects on incubation behavior, nest desertion, and care of young. Techniques for investigating the effects of chemicals on these behaviors are in their infancy and, at present, are prohibitively expensive. Agency research has been proposed to investigate methods of incorporating natural incubation into future reproduction guidelines at a reasonable cost.

8. Required Analyses

a. Statistical

The statistical analysis of avian reproduction studies typically has been analysis of variance (Heath and Spann 1973, Heinz 1976a, US EPA 1978a, ASTM 1979a) using the

parameters set forth in this guideline. Angular transformations have been used to a lesser extent (Heath et al. 1972b). Regression analysis is a powerful tool that may be used if there are data at sufficient dose levels to obtain a dose-response curve. Regression analysis has been recommended, as an option by ASTM (1979a), and methods directed toward regression analysis are being developed (Stromberg, personal communication). It is the opinion of the author of this test guideline that regression analysis is a more useful tool than currently typical methods because it yields a dose response curve and this curve can be used for extrapolation. Analysis of variance provides only significance at a particular level and does not lend itself to extrapolation on a reliable basis. At the present time, methods and background work have not been developed for a test oriented primarily towards a dose-response curve. More dose levels would be needed, possibly with fewer animals per level. This subject may be included in the Agency's research projects. In the meantime, this guideline uses methods appropriate to analysis of variance.

b. Test Substance Concentrations

Samples of treated diets will be analyzed to determine the actual levels to be used in the test. Analysis will help to detect mathematical errors in calculating concentrations, technicians' errors in mixing diets, and manufacturers' errors in determining the amount of active ingredient in a test substance (Heinz, personal communication). All test substance concentrations will be analyzed so that, even with only three test concentrations, a dose-response curve (if obtained) would be based on

measured concentrations, rather than on normal levels. A second analysis about midway through the test will corroborate initial levels.

c. Basal Diet

Most testing facilities use diets prepared by commercial feed companies. Some facilities may have a commercial company prepare a diet to order. Normally, such diets are supplied with a quantitative list of ingredients, and such a list should be supplied with the test report. If there are supplements added to the diet, a list of all supplemental ingredients also should be submitted. Analysis of ingredients in the basal diet is important because there are a number of potential test substances, such as certain metals, that may interact with components of the diet and possibly affect the results of a test. A nutrient analysis will allow for a better evaluation of such results. In addition, it is possible that dietary deficiencies or imbalance of ratios of nutrients also could affect the results. Even though commercial companies normally supply a nutritionally adequate diet, it is important to know the components because no rigid requirements exist for the type and constitution of the diet used.

9. Acceptability Criteria

Test acceptability is dependent upon following mandatory requirements and having acceptable control productivity and survival. When control values do not reach the typical observed values, as given in section (h)(11) of the test guideline, it is very likely that there are problems with some aspect of the test. Since reproductive parameters of treated birds are compared with controls, it is essential

that values for control birds are normal as determined from similar reproduction studies.

Rather than requiring every parameter to achieve normal values, it was decided that acceptability of a test should be based on the final parameter of 14-day old survivors per control hen as one criterion. For bobwhite, the requirement of 12 survivors per hen over a ten week period was selected because data in the Agency's pesticide files show that acceptable tests have reached and usually surpassed this level. Heath and Spann (1973) passed these levels in test birds over eight weeks, and had comparable control productivity of 7.5 survivors per hen in four weeks. Heath et al. (1972b) reported 11.5 control survivors per hen in eight weeks.

Eggshell thickness of control birds is also a criterion of test acceptability because thin eggshells among control birds are usually a sign of inadequate diet, which in turn could affect other aspects of the test. Heath et al. (1972b) reported bobwhite shell thickness of control groups as .207 mm. Heath and Spann (1973) reported control values as ranging from .22 to .25 mm. McGinnis et al. (1976) reported average bobwhite values of .226 and .236 mm (ranges-.196-.267 and .185-.274, respectively) in a non-test investigation. It might be unrealistic to expect all eggs to meet the designated thickness of .19 mm, but if average thickness is less, then a problem probably exists.

A well conducted test with adult birds should not result in any but an occasional mortality in control groups, even though the test is relatively long. This is especially true since this guideline provides for rearrangement of

incompatible birds during the acclimation period. Therefore, control mortality in excess of 10% indicates problems with some aspect of the test.

C. Test Conditions

1. Test Species

a. Selection

The bobwhite, Colinus virginianus (L), is the species to be tested. The choice of bobwhite is based on several factors. Although no single species would satisfy all criteria for species selection, the bobwhite has a number of favorable attributes. The bobwhite has a widespread distribution throughout much of the United States, and it is an important part of the avifauna of the southern U.S. It occurs in a variety of terrestrial habitats and is likely to be exposed to toxic substances that occur in such habitats. As an ecologically relevant species, the bobwhite represents its own species as well as serving as an indicator for other species.

The bobwhite has not been used extensively in reproduction tests that have been published. In general, waterfowl are considered to be more susceptible to reproductive impairment than gallinaceous birds. However, DeWitt (1962) found bobwhite to be 50 times more sensitive than mallards to reproductive impairment from toxaphene. Similarly, gallinaceous birds have exhibited reproductive impairment from PCBs, but mallards have not (Stickel 1975). Other confidential data in the Agency's pesticide files have confirmed that bobwhite are sensitive to some chemicals although not as frequently as are waterfowl. When reproductive tests are conducted on both gallinaceous birds

and waterfowl, this combination of species will often indicate impairment that might not be found if only one species were tested.

The bobwhite is also amenable to testing in the laboratory. Bobwhite can be bred in captivity and are readily available from commercial sources so that testing of this species will not deplete wild stocks. There is sufficient information on the nutritional, habitat, and behavioral characteristics of natural populations of bobwhite in order to meet the basic nutritional and physical requirements of the species in the laboratory.

A major advantage of reproduction testing with bobwhite instead of other galliforms is that bobwhite is typically one of two species used in short-term toxicity tests with birds. There is an advantage in using the same species in reproductive tests that also can be used in short-term test or field tests. Bobwhite have been used for short-term laboratory test and actual or simulated field tests from the beginnings of ecological effects testing (DeWitt et al. 1962) to the present (US EPA 1980b). Thus, the choice of bobwhite as a test species facilitates comparisons of the results from different kinds of tests.

In addition, bobwhite are generally considered to have a positive economic value. Although the Agency is charged with the protection of all species in the environment, the choice of an economically valuable species for testing is appropriate to the cost-benefit or risk-benefit analyses upon which Agency decisions frequently are based.

If a test is to simulate toxicity to naturally occurring populations of bobwhite, then it is important to use birds that are phenotypically indistinguishable from wild birds. Since many chemicals act upon specific enzymes and enzymes are based on the genetic code, the use of birds genotypically similar to wild birds would be desirable. However, the determination of phenotype is a simple observational process, whereas genotypic determination is impractical, if possible at all. In addition, wild birds have a degree of heterogeneity that would not be typical of any given genotype.

The necessity for using healthy birds is obvious since the test is designed to determine toxicity to typical populations. It is admitted that not all birds occurring in natural populations are healthy, but the majority of survivors in natural environments are healthy. The health of birds is also important in reducing the number of variables that limit comparisons between tests. There are several checks in this test guideline that help to insure that healthy birds are used. The use of previously untested birds not selected for resistance and being from disease free flocks provides a basically healthy stock. Visual observations select out abnormal or unhealthy birds from that stock. A final check on health is based on the birds ability to survive two weeks immediately preceding exposure. The 3% maximum mortality during this period allows for an occasional death that may occur during acclimation at the time when unfamiliar birds in a pen are becoming adapted to each other.

The choice of first year birds was made in this guideline primarily because it facilitates comparisons with existing data and data that may be generated according to the US EPA (1978a) protocol for bobwhite. It also ensures that birds will be the same age; whereas if no age were specified, birds in the same test could be various ages. Use of first year birds also reduces the cost of birds and ensures that test birds will not have been used in previous reproduction tests.

2. Maintenance of Test Species

a. Acclimation

An acclimation period is necessary for birds to become familiar with the test environment. Ideally, birds will be maintained in test cages for several months, but this is impractical for testing facilities that purchase adult birds. It is also sometimes necessary to alter the composition of birds in a pen because of excessive aggression or other incompatibilities. The acclimation period allows time for rearranging incompatible birds.

b. Diet

There are few data on the detailed nutritional requirements for bobwhite. This subject is being investigated under a current contract and is a proposed research need. At the present time, a commercial game bird breeder ration is recommended. All known testing facilities in this country use a commercial ration or a similar but specially prepared ration made by a commercial company. There are no known data to support an alternative diet for bobwhite. Changes may be made in the future when additional data become available. The recommendation of a commercial

game bird starter ration is made for the same reasons.

Samples of treated diets will be analyzed to determine the actual test concentrations used in the test. See section 2.2.8 for details

3. Facilities

Very few bobwhite reproduction tests have been reported in the literature, although a number have been submitted to the Agency and classified as confidential. As a result, it is difficult to define optimal conditions and most conditions have been recommended, rather than required. Heath et al. (1972b) and Heath and Spann (1973) used outdoor pens with wire mesh floors for their bobwhite reproduction studies. Heath and Spann (1973) specified the size of cages as 3 X 6 feet; they housed 6 hens and 3 cocks in each pen. Heath et al. (1972b) may or may not have used the same pens; they housed 3 hens and 2 cocks in each pen. Kendall et al. (1978) used pairs of bobwhite housed in quail battery breeding pens (size unspecified) indoors, apparently at 24°C. They also used .6 x 1.5m (2 x 5 ft) pens, housing 5 hens and 5 cocks, in a poultry house. Illumination intensity, temperature, and humidity were not reported by Heath et al. (1972) and Heath and Spann (1973). Presumably, these were not controlled since pens were outdoors.

US EPA (1978a, 1980b) and ASTM (1979a) have both developed protocols for avian reproduction tests. Rationales for selecting particular conditions were not spelled out in either protocol. Reasons were apparently based on experience with reproduction tests that have been submitted to the Agency for pesticide registration and that have been classified as confidential. These three protocols

have specified size only as being "adequate". All three protocols recommend 21°C, 55% relative humidity, and adequate ventilation. The same conditions have been recommended in this guideline, since these protocols are the most widely used and data developed from this guideline will be comparable with data gathered using all three protocols.

Galvanized metal is the recommended construction material. This material has been widely used in toxicity tests (Heath et al. 1972a, Hill et al. 1975) and has been recommended in ASTM (1979a) and EPA (1978a) protocols for reproduction tests. The ASTM (1979a) protocol suggests stainless steel or perfluorocarbon plastics (e.g., Teflon) as alternatives. As a relatively non-adsorbent material, perfluorocarbon plastics may be used to coat wood or other materials that might be contaminated by chemicals if used uncoated. Because of the tendency of wood to sorb chemicals, uncoated wood may not be used where it is likely to become contaminated and come in contact with birds in subsequent tests.

Pens should be cleaned and sanitized between tests. Brushing and/or steam cleaning appear to be the most appropriate since they do not involve the use of chemicals that could affect subsequent tests. Detergents and bleach have been used by Denver Wildlife Research Center (Tucker, personal communication) and Patuxent Wildlife Research Center (Heinz, personal communication). The use of chemical disinfectants, such as quaternary ammonium compounds, should be avoided because of possibility that these compounds can leave toxic residues. However, the widely used cold sterilization method with ethylene oxide is acceptable, if

needed for disease control. Pens should not be cleaned during a test in order to minimize disturbance to the test birds.

4. Environmental Conditions

a. Temperature (See Section II.C.3)

b. Humidity (See Section II.C.3)

D. Reporting

The information that is required to be reported in section II.B.8.b is essential to a proper evaluation of the test results. These required items are needed (1) to establish that the test was conducted according to specifications, (2) to evaluate those conditions and procedures that could affect the results of the test, and (3) to supply the Agency with sufficient information to conduct and independent analysis of statistics and conclusions. The location of the raw data storage will allow the Agency to find additional information that may have been left out of the report or that may be needed for enforcement purposes. The location is necessary because some chemical companies request the testing facility to keep these data, while other companies keep their own. This information is needed in a detailed manner because this avian reproduction guideline contains few rigid requirements. Even when minimums or maximums are specified, it is important to know how much the test may have exceeded specifications, such as; if test birds were observed more frequently than required, if the number of test concentrations exceeded the three levels required, etc.

The information required in section II.B.8.b on Test Substance Concentration to be available, but not included in

the test report, may be needed if there are serious concerns about the results or validity of the test. This information will not normally be needed and therefore is not required routinely.

III. Economic Aspects

Three laboratories were surveyed to estimate costs for performing the test outlined in this guideline. The individual laboratories gave prices of $17,000, $10,780, and $38,000. The "best estimate" based upon the survey was $21,927. A cost estimate also was made by separating the protocol into components and estimating the cost of each component, including direct labor cost, overhead cost, other direct cost, general and administrative cost, and fee. The best estimated final cost, based upon this calculation method, was $20,562, with an estimated range of $10,281 to $30,844 based on ± 50% of the best estimate. The calculated estimate is similar to the best estimate based on the survey. Marked differences in prices obtained from the different laboratories may have resulted from a number of factors, such as nature of the chemical, overhead rates, outside consultants, automation, marketing strategies, and other factors as outlined in a cost analysis report by Enviro Control (1980). The cost estimates were made assuming that the requirements of the Good Laboratory Practice Standards, as specified in section (d) of the Bobwhite reproduction guideline, are being satisfied.

In a cost analysis of subpart E pesticides guidelines, laboratories were surveyed in 1978 and in 1980 to determine the cost of testing (US EPA 1980a). The cited costs did not differentiate between species, however, the unit cost for an avian reproduction test was $24,000 in 1978 and $28,000 in 1980.

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EG-17
August, 1982

MALLARD REPRODUCTION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MALLARD REPRODUCTION TEST

(a) Purpose. This guideline is designed to develop data on the reproductive effects on the mallard of chemical substances and mixtures subject to chronic environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003 15 U.S.C. 2601 et. seq.). The Agency will use these and other data to assess the reproductive effects on birds that these chemicals may present in the environment.

(b) Definitions. (1) The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792--Good Laboratory Practice Standards apply here. In addition, the following definitions apply generally to this guideline:

(i) "Acclimation" Physiological and behavioral adaptation to environmental conditions (e.g., housing and diet) associated with the test procedure.

(ii) "Test substance" The specific form of a chemical or mixture of chemicals that is used to develop the data.

(iii) "Photoperiod" The light and dark periods in a 24 hour day. This is usually expressed in a form such as 17 hours light/ 7 hours dark or 17L/7D.

(iv) "Basal diet" The untreated form of the diet, such as the diet obtained from a commercial source.

(2) The definitions in this section refer specifically to the production and quality of eggs and the subsequent development of these eggs up to the point where young are 14 days old.

(i) "Eggs laid" This term refers to the total egg production during the test, which normally includes ten weeks of laying. Values are expressed as numbers of eggs per pen per season (or test).

(ii) "Eggs cracked" Eggs determined to have cracked shells when inspected with a candling lamp. Fine cracks cannot be detected without using a candling lamp and if undetected will bias data by adversely affecting embryo development. Values are expressed as a percentage of eggs laid by all hens during the test.

(iii) "Eggs set" All eggs placed under incubation, i.e., total eggs minus cracked eggs and those selected for analysis of eggshell thickness. The number of eggs set, itself, is an artificial number, but it is essential for the statistical analysis of other development parameters.

(iv) "Viable embryos (fertility)" Eggs in which fertilization has occurred and embryonic development has begun. This is determined by candling the eggs 14 days after incubation

has begun. It is difficult to distinguish between the absence of fertilization and early embryonic death. The distinction can be made by breaking out eggs that appear infertile and examining further. This distinction is especially important when a test compound induces early embryo mortality. Values are expressed as a percentage of eggs set.

(v) "Live 21-day embryos" Embryos that are developing normally after 21 days of incubation. This is determined by candling the eggs. Values are expressed as a percentage of viable embryos (fertile eggs).

(vi) "Hatchability" Embryos that mature, pip the shell, and liberate themselves from their eggs on day 25, 26, or 27 of incubation. Values are expressed as a percentage of viable embryos (fertile eggs).

(vii) "14-day old survivors" Birds that survive for two weeks following hatch. Values are expressed both as a percentage of hatched eggs and as the number per pen per season (test).

(viii) "Eggshell thickness" The thickness of the shell and the membrane of the egg at several points around the girth after the egg has been opened, washed out, and the shell and membrane dried for at least 48 hours at room temperature. Values are expressed as the average thickness of the several measured points in millimeters.

(c) Test Procedures--(1) Summary of test. (i) After birds have been obtained they should be observed for health and acclimated for at least two weeks.

(ii) Test birds should be randomly assigned to control and various treatment groups.

(iii) The test substance should be thoroughly and evenly mixed into the diet at concentrations specified in the test rule. All treatment levels should be analyzed for test substance concentrations at the beginning and midway through the test.

(iv) Birds should be weighed at the beginning of the test, at 14-day intervals until the onset of laying, and at termination of the test.

(v) Photoperiod should be carefully controlled on a short-day basis during the initial exposure phase, then increased to 16-17 hours to induce egg laying.

(vi) Birds should be observed regularly for abnormal behavior or mortality throughout the test.

(vii) Eggs should be removed daily and stored until there is a sufficient quantity for incubation. All eggs should be candled for cracks and cracked eggs removed. Once every two weeks, all eggs produced that day should be analyzed for eggshell thickness. Incubated eggs should be candled on day 14 and day 21. Hatching should be completed by day 27.

(viii) Hatchlings should be maintained pens until they are 14 days old. Abnormal behavior or death should be reported. Ducklings should be weighed on day 14.

(ix) A statistical analysis should be performed, preferably by analysis of variance or regression analysis.

(x) The report should include all conditions, procedures, and results. Data should be sufficiently detailed for an independent statistical analysis.

(xi) All treated birds should be sacrificed and disposed of properly. Control birds may be kept as breeding stock, but should not be used in any other tests. Control offspring may be reared and used in another test as adults.

(2) [Reserved]

(3) [Reserved]

(4) Definitive test--(i) Test substance. (A) The concentrations of test substance in the diet will be specified in the test rule. Generally, three treatment groups and a control group will be used. The higher two treatment levels will be multiples (often 5x, 10x, or 20x) of the lowest treatment level. The highest treatment levels usually will be below lethal levels, unless predicted exposure levels are high enough to approximate lethal levels.

(B) The material to be tested should be analytically pure and the degree of purity should be reported along with the percentage of each impurity at levels specified in the test rule. If specifically required by a test rule for a particular substance or mixture, the technical grade should be tested. The test rule will specify the degree of purity or a range of compositions of the technical grade material.

(ii) Controls. A concurrent control is required during every test. The control birds should be from the same hatch as the test groups. Control and test birds should be kept under the same experimental conditions. The test procedures should be the same for control and treated birds, except that no test substance should be added to the diets of control birds. If a carrier is used in preparation of the test diets, the same carrier should be added to the diets of control birds in the highest concentration used for test diets. The use of shared controls is acceptable for concurrent tests as long as the same carrier is used for all the tests.

(iii) Test groups and numbers of birds. (A) Either one of two designs may be used for numbers of animals and pens. For one design, each of the three treatment groups and the control group should consist of a minimum of 8 replicate pens, with each pen containing one male and three females. For the alternative

design, each group should consist of 12 replicate pens containing one male and one female per pen; the use of 20 replicate pens in the control group may yield a test with greater statistical power. Either design is acceptable as long as productivity reaches the definitive values given in paragraph (c)(4)(xii) of this section. Testing facilities using an experimental design with which they are not familiar are advised to experiment first without test substances in order to determine the feasibility of obtaining acceptable productivity levels.

(B) All control and treatment birds should be randomly distributed to pens from the same population.

(iv) Duration of test. (A) The test consists of three phases following acclimation to test facilities. The initial phase begins with exposure of treatment groups to diet containing the test substance and is typically six to eight week long. After the initial phase, the photoperiod is manipulated according to paragraph (c)(4)(v) of this section to bring the hens into laying condition. This second phase ends with the onset of egg-laying and is typically two to four weeks long. The final phase begins with the onset of laying and lasts for at least eight weeks, preferably ten weeks. A withdrawal study period may be added to the test phase if reduced reproduction is observed. The withdrawal period, if used, need not exceed three weeks.

(B) Exposure of adult birds to the test substance should be continuous throughout the test. Unless otherwise specified in the test rule, test birds should be exposed to the test substance for at least ten weeks prior to the onset of egg laying.

(v) Preparation for reproduction (photoperiod). (A) Lighting regimes (photoperiod) are critical to successful reproduction. Various photoperiod regimes have been demonstrated to give acceptable results. Any photoperiod regime that results in productivity that meets the definitive values given in paragraph (c) (4)(xii) of this section is acceptable as long as birds are exposed to treated diets a minimum of ten weeks prior to the onset of laying. Regardless of the method selected, lighting should be controlled carefully. It is important during the initial phase to not interrupt the dark period unless absolutely necessary.

(B) A suggested photoperiod regime would consist of maintaining birds under a photoperiod of seven or eight hours of light during the initial phase. At the end of the initial phase, the photoperiod may be increased to 16-17 hours of light per day. The photoperiod may be maintained at this level for the remainder of the study, although an increase each week of 15 minutes per day is acceptable.

(vi) Observations of record on adult birds. (A) Body weights should be recorded for each adult bird at the beginning of the treatment period, at 14-day intervals until the onset of egg laying, and at termination of treatment. Birds may be weighed during egg production phase of the study only if they are not unduly stressed by the procedure. Food consumption should be measured and recorded by pen at least as often as body weights are measured prior to the onset of laying and at least bi-weekly throughout the rest of the study.

(B) Observations on adult birds should be made at least once a day. Any mortality or other signs of toxicity should be described and recorded by date or day of test. Gross pathological examinations should be conducted on all birds that die during the test period, and for all survivors at the end of the test. Analysis for test substance residues of two or more tissues (e.g., muscle, fat) is encouraged, but not required (unless specified in the Test Rule).

(vii) Egg collection, storage, and incubation. All eggs should be collected daily, marked according to the pen from which collected, and should be stored at 16°C and 55-80 percent relative humidity. Storage in plastic bags may improve uniformity of hatching. Stored eggs should be turned daily. At weekly or bi-weekly intervals, eggs should be removed from

storage and be candled to detect eggshell cracks. Except for eggs with cracked shells and those eggs removed for eggshell thickness measurements all eggs should be set after candling for incubation in a commercial incubator. If incubators are not equipped to automatically turn eggs, they should be turned daily by hand. During the incubation period, eggs should be maintained at 37.5°C and approximately 70 percent relative humidity. Eggs should be candled again on day 14 of incubation to determine fertility and early death of embryos. A final candling should be done on day 21 to measure embryo survival. On day 23, eggs should be removed to a separate incubator or hatcher. Hatching will normally be complete by the end of day 27.

(viii) Duckling maintenance. By day 27 of incubation, the hatched mallard ducklings should be removed from the hatcher or incubator. Ducklings should be either housed according to the appropriate parental pen group or individually marked (such as by leg bands) as to parental group and housed together. Ducklings should be maintained in commercial brooder pens or pens of similar construction. Pens should be constructed of galvanized metal or stainless steel. Temperature in the pens should be controlled, preferably by a thermostatic control device. A temperature gradient in the pen from approximately 35°C to approximately 22°C will allow young birds to seek a proper

temperature. Temperature requirements for young birds typically decline over this range from birth through the first several weeks of life. Ducklings should be provided a standard commercial duck starter ration, or its nutritional equivalent. No test substance may be added to the diets of ducklings. Ducklings should be maintained until they are 14 days old.

(ix) Observations of record on ducklings. The hatchability, percentage of normal hatchlings, percentage of 14-day old survivors, and number of 14-day old survivors per hen should be recorded and reported. Ducklings should be observed daily from hatching until they are 14 days old. Mortality, signs of toxicity, and other clinical abnormalities should be recorded at least cumulatively through day 5 and recorded by age from days 5 through 14. Average body weights should be recorded for ducklings at day 14.

(x) Eggshell thickness. Once every two weeks all eggs newly laid that day should be removed and measured for eggshell thickness. Eggs should be opened at the girth (the widest portion), the contents washed out (or used or saved for egg residue analysis), and the shells air dried for at least 48 hours. The thickness of the shell plus the dried membrane should be measured at a minimum of 3 points around the girth using a micrometer calibrated at least to 0.01 mm units.

(xi) Typical observed values. The values reported here represent those observed from a few testing facilities under their conditions. These values are not necessarily representative of those from all facilities, however, if a reproduction test does not meet or at least approach these values for control birds, then there is likely to be a problem with test procedures or conditions that should be investigated and corrected. Typical values include:

(A) Eggs laid. Normal values for mallards - 28 to 38 eggs per hen per season.

(B) Eggs cracked. Normal values for mallards - 0.6 percent to 6 percent of eggs laid.

(C) Viable embryos (fertility). Normal fertility values for mallards - 85 percent to 98 percent of eggs set.

(D) Live 21-day embryos. Normal values for mallards - 97 to 99 percent of viable embryos.

(E) Hatchability. Normal values for mallards - 50 percent to 90 percent of viable embryos (fertile eggs).

(F) 14-day-old survivors. Normal values for mallards - 94 percent to 99 percent of eggs hatched.

(G) Eggshell thickness. Normal average values for mallards - 0.34 mm to 0.39 mm.

(xii) Definitive test criteria. (A) A test is unacceptable if mallard duckling productivity in control groups does not average fourteen 14-day old survivors per hen over a ten week period.

(B) A test is unacceptable if the average eggshell thickness in control groups is less than 0.34 mm.

(C) A test is unacceptable if more than 10% of the adult control birds die during the test.

(5) [Reserved]

(6) Analytical measurements--(1) Statistical analysis. (i) Experimental groups should be individually compared to the control group by analysis of variance. Other accepted statistical methods may be used as long as they are documented and described. In particular, regression analysis is highly desirable if the data and number of dose levels allow the use of this technique. Sample units are the individual pens within each treatment level or control. Analysis should include:

(A) Body weights of adults.

(B) Food consumption of adults.

(C) Percentage of hens laying eggs. This should always be determined when pens contain a single pair; if feasible, it should be determined when pens contain groups.

(D) Number of eggs laid per pen.

(E) Percentage of cracked eggs.

(F) Percent viable embryos of eggs set.

(G) Percent live 21-day embryos of viable embryos.

(H) Percent hatching of viable-embryos.

(I) Percentage of hatchlings that are normal.

(J) Percent 14-day-old survivors of normal hatchlings.

(K) Number of 14-day-old survivors per hen.

(L) Body weights of 14-day-old survivors.

(M) Eggshell thickness.

(ii) Analysis for Test Substance Concentrations. (A) Samples of treated diets should be analyzed to confirm proper dietary concentrations of the test substance. If samples cannot be analyzed immediately, they should be stored appropriately (e.g., frozen at a tempererature of -15°C or lower) until analysis can be performed. Analyses should be conducted on all test substance concentrations at the beginning of the test period and again 10 to 12 weeks later. If not otherwise available, data should be generated to indicate whether or not the test substance degrades or volatilizes. If the test substance is known or found to be volatile or labile to the extent that 25 percent or more loss occurs within one week, then test substance diets should be prepared (freshly or from frozen concentrate) at a frequency that will prevent more than 25 percent loss of test substance.

(B) The assay method used to determine actual concentrations should be reported according to paragraph (e)(1)(vi) of this section.

(C) Analysis of basal diet. A nutrient analysis of the basal diet should be included in the test report. For commercially prepared basal diets, the list of ingredients supplied by the manufacturer is normally sufficient, if it is detailed. The composition of any vitamin or other supplements should also be reported.

(d) Test conditions--(1) Test species (i) Selection. (A) The mallard, Anas platyrhynchos L., is the test species. Test birds should be pen-reared. They may be reared in the laboratory or purchased from commercial breeders. Rearing stock and/or test birds should be obtained only from sources that have met the requirements for "U.S. Pullorum-Typhoid Clean" classification. Birds should be obtained only from sources whose colonies have known breeding histories. If possible, a history of rearing practices for test birds should be obtained and made available upon request. This history should include lighting practices during rearing, disease record, drug and any other medication administered, and exact age. Test birds should be phenotypically indistinguishable (except for size) from wild stock. Conscientious breeders of such birds will periodically

outbreed their flocks with genetically wild stock in order to maintain a genetic composition that approximates the heterogeneity of naturally occurring birds.

(B) All control and experimental birds used in a test should be from the same source and strain. If shipped, all birds should be examined following shipment for possible physical injury that may have occurred in transit. All birds should have a health observation period of at least two weeks prior to selection for treatment. Birds should be in apparent good health. Deformed, abnormal, sick, or injured birds should not be used. A population of birds should not be used if more than 3 percent of either sex die during the health observation period. Birds should not have been selected in any way for resistance to toxic substances. Birds should not have been used in a previous test, either in a control or treatment group. Offspring of birds used in a treatment group in a previous test should not be used, but offspring of birds used as a control in a previous test are acceptable.

(C) Test birds should be approaching their first breeding season and should be at least seven months old. All test birds should be the same age within one month. The age of test birds should be reported.

(D) Mallards should be acclimated to test facilities and untreated basal diet for at least two weeks. Acclimation may be in the actual pens used in the test or in identical pens. The acclimation period may coincide with the health observation period. Birds should be randomly assigned to treatment and control pens. However, when birds in a pen are incompatible, they may be rearranged within a control or treatment group at any time prior to initiating treatment.

(E) During holding, acclimation, and testing, birds should be shielded from excessive noise, activity, or other disturbance. Birds should be handled only as much as is necessary to conform to test procedures.

(ii) Diet--(A) Adult birds. (1) A standard commercial duck breeder ration, or its nutritional equivalent, should be used for diet preparation. This ration or basal diet should be used for both control and treatment birds and should be constant throughout the duration of the study. Antibiotics or other medication should not be used in the diet or water of breeding birds. It may not be possible to obtain food that is completely free of pesticides, heavy metals, and other contaminants. However, diets should be analyzed periodically for these substances and should be selected to be as free from contaminants as possible. A nutrient analysis (quantitative list of

ingredients) of the diet should be included with the test report.

(2) The test substance should be mixed into the diet in a manner that will ensure even distribution of the test substance throughout the diet. If possible, the test substance should be added to the diet without the use of a carrier or diluent. If a diluent is needed, the preferred diluent is distilled water; but water should not be used for test substances known to hydrolyze readily. When a test substance is not water soluble; it may be dissolved in a reagent grade evaporative diluent (e.g., acetone, methylene chloride) and then mixed with the test diet. The solvent should be completely evaporated prior to feeding. Other acceptable diluents may be used, if necessary, and include table grade corn oil, propylene glycol, and gum arabic (acacia). If a diluent is used, it should comprise no more than 2% by weight of the treated diet, and an equivalent amount of diluent should be added to control diets.

(3) Diets may be mixed by commercial or mechanical food mixers. Other means are acceptable as long as they result in even distribution of the test substance throughout the diet. Screening of the basal diet before mixing is suggested to remove large particles. For many test substances, it is recommended that diets be mixed under a hood. Frequently, the test substance is added to an aliquot of the basal diet to form a premix

concentrate. The premix concentrate should be stored so as to maintain the chemical concentration. For final preparation of test diets, the premix is mixed with additional basal diet to form the proper concentrations. The frequency with which final treated diets are prepared will depend upon the stability and other characteristics of the test substance. Unless otherwise specified in the test rule or determined by degradation or volatility studies, it is recommended that final diets be prepared weekly, either fresh or from a concentrate. For volatile or labile test substances, test diets should be mixed frequently enough so that the concentrations are not reduced from initial concentrations by more than 25 percent. Analysis of diets for test substance concentrations is required as specified in paragraph (c)(6)(ii).

(4) Clean water should be available ad libitum. Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water should be changed daily or more often.

(B) Young birds. Young birds produced during the test should be fed a commercial duck starter ration, or its nutritional equivalent. No test substance should be added to the diets of young birds. No antibiotics or medication should be used in the diet.

(2) Facilities. (i) Mallards should be housed in breeding pens or cages of adequate size conforming to good husbandry practices. Space requirements for mallards have not been well defined, but it is recommended that there be at least 10,000 square centimeters (approximately 5.4 square feet) of floor space per bird. Documentation that reproductive parameters and health of birds are not adversely affected should be provided for cages much smaller than this area. The preferred construction materials are stainless steel, galvanized sheeting, and wire mesh. For enclosed cages, floors and external walls may be wire mesh; ceilings and common walls should be solid sheeting. Open-topped pens may be constructed of the same materials for the side walls with open tops and wire mesh or concrete floors. Concrete floors should be covered with litter such as straw, wood shavings, or sawdust. Other construction materials, except wood, are acceptable if they can be kept clean. Wood may be used as vertical framing posts for the support of wire mesh or for horizontal framing along the top of the pen. Wood should not be used for floors or lower sides of pens unless it has been coated with a non-adsorbent material such as perfluorocarbon plastic (e.g., Teflon) or unless the wood is replaced between tests.

(ii) Pens should be disassembled (if feasible) and should be cleaned thoroughly between tests. Steam cleaning of enclosed

cages is recommended. Enclosed cages may be brushed thoroughly, as an alternative method. For open-topped pens, the sides and vertical supports should be thoroughly brushed. Any used floor litter should be discarded. The floor composition will dictate methods used to clean the floor. If litter is used on the floor, it should be fresh and clean when birds are placed in the pen. The use of detergents or bleach is acceptable, but other chemical disinfectants (such as quaternary ammonium compounds) should not be used. When necessary to control disease vectors, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended.

(iii) Pens should be kept indoors in order to better control lighting, temperature, humidity, and other factors. Outdoor pens may be used only during the normal breeding season. The photoperiod should be carefully controlled, preferably by automatic timers. A 15-30 minute transition period is desirable. The photoperiod regime is described under paragraph (c)(4)(v). Lights should emit a spectrum simulating that of daylight. The use of shorter wave-length "cool-white" fluorescent lights that do not emit the daylight spectrum should be avoided. Illumination intensity should be about 6 foot-candles at the level of the birds.

(iv) Temperature and humidity should be controlled during the study. Recommended levels are 21°C and 55 percent relative humidity. Temperature for indoor tests should be recorded at least weekly at the same time of day and should be reported. For tests conducted without temperature control, temperature minimums and maximums should be recorded daily. Continuous temperature monitoring is desirable. Temperature recording should be made at level of 2.5-4 cm above the floor of the cage. Recording of approximate humidity levels is also desirable. Good ventilation should be maintained. Suggested ventilation rates are 4 changes per hour in winter and 15 changes per hour in the summer.

(v) If facilities are being used for the first time, it may be desirable to allow birds to breed in the facility prior to testing in order to ensure that controls will have acceptable productivity according to the requirements given in paragraphs (c)(4)(xi) and (xii)].

(3) [Reserved]

(e) Reporting. (1) The test report should include the following information:

(i) Name of test, sponsor, test laboratory and location, principal investigator(s), and actual dates of beginning and end of test.

(ii) Name of species tested (including scientific name), age of birds (in months) at the beginning of the test, source of birds, and body weights for adult birds throughout the test.

(iii) Description of housing conditions, including type, size, and material of pen, temperature, humidity, photoperiod and lighting intensity, and any changes during the test.

(iv) Detailed description of the basal diet, including source, composition, diluents (if used), and supplements (if used). A nutrient analysis of the basal diet should be included.

(v) Detailed description of the test substance including its chemical name(s), source, lot number, composition (identity of major ingredients and impurities), and known physical and chemical properties pertinent to the test (e.g., solubility, volatility, degradation rate, etc.).

(vi) The number of concentrations used, nominal and measured concentrations of test substance in each level, assay method used to determine actual concentrations, storage conditions and stability of treated diets, number of birds per pen and number of replicate pens per concentration and for controls.

(vii) Acclimation procedures and methods of assigning birds to test pens, including method of randomization, and any rearrangements due to incompatibility.

(viii) Frequency, duration, and methods of observation.

(ix) Description of any signs of intoxication, including time of onset, duration, severity (including death), and numbers affected, including accidental deaths or injuries.

(x) Food consumption per pen and any observations of repellency or food palatability.

(xi) Method of marking all birds and eggs.

(xii) Details of autopsies.

(xiii) Egg and hatching data in summary and by pen per week in sufficient detail to allow an independent statistical analysis. Data should be presented for all of the parameters listed in paragraph (c)(6)(i). The number of eggs set also should be reported.

(xiv) Egg storage, incubation, and hatching temperatures, relative humidities, and turning frequencies.

(xv) Observations of health and weights of young at 14 days of age.

(xvi) Location of all raw data storage.

(xvii) Methods of statistical analysis and interpretation of results.

(xviii) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(2) In addition, the following information should be available upon request:

(i) A general description of the support facilities.

(ii) A description of the Quality Control/Quality Assurance program, including the Average Quality Level for the program element performing the test, procedures used, and documentations that these levels have been achieved.

(iii) The names, qualifications, and experience of personnel working in the program element performing the test, including the study director, principal investigator, quality assurance officer, as well as other personnel involved in the study.

(iv) Standard operating procedures for all phases of the test and equipment involved in the test.

(v) Sources of all supplies and equipment involved in the test.

(vi) Diagram of the test layout.

(vii) Originals or exact copies of all raw data generated in performing the test.

(viii) A detailed description, with references, of all statistical methods.

ES-14
August, 1982

TECHNICAL SUPPORT DOCUMENT

FOR

MALLARD REPRODUCTION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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TEST SUPPORT DOCUMENT FOR MALLARD REPRODUCTION TEST

I. Purpose

The purpose of this document is to provide the scientific background and rationale used in the development of Test Guideline EG-17 which uses Mallards to evaluate the effect of chemical substances on reproduction. The Document provides an account of the scientific evidence and an explanation of the logic used in the selection of the test methodology, procedures and conditions prescibed in the Test Guideline. Technical issues and practical considerations are discussed. In addition, estimates of the cost of conducting the test are provided.

II. Scientific Aspects

A. General

The earliest investigations of the effects of chemicals on reproduction of native birds were in the 1950s (DeWitt 1956, Genelly and Rudd 1956). Chemicals were administered in the diet, but procedures varied. Laboratory investigations of reproductive effects of pesticides continued at Patuxent Wildlife Research Center, but methods were not reported well (DeWitt et al. 1962, DeWitt et al. 1963). In 1964, a very brief protocol for reproduction studies was developed at that center (Stickel and Heath 1965). This protocol outlined the egg parameters to be studied. Ratcliffe (1967), in a classic paper, correlated the decline of certain avian populations with thin eggshells that apparently had resulted primarily from exposure to DDT and DDE. Heath et al. (1969) presented the first clearcut experimental data showing that DDE caused thin eggshells in mallards. The protocol used by Heath et al. (1969),

although not fully detailed, formed the basis for most future avian toxicological reproduction studies. In 1968, USDA developed a guideline for evaluating reproductive effects of toxic chemicals to birds (US EPA 1975). This guideline apparently was developed in conjunction with Patuxent personnel as it bears a great similarity to methods used at Patuxent, but was available prior to the publication of Heath et al. (1969). The Agency (US EPA 1975) developed composite protocols for reproduction tests from the limited published information and unpublished information, especially the USDA protocol which was presented as an exhibit. No complete, suitable protocol for bobwhite was available from the published literature at that time. More, but insufficient, literature was available for mallards, particularly as relates to eggshell thinning (US EPA, 1975) since Heath et al. (1969, 1972b) and Heath and Spann (1973) had published the results of mallard reproduction studies. The Agency's pesticide guidelines were revised (US EPA 1978a), but the basic method of the earlier guidelines for reproduction tests was retained. There have been few mallard reproduction tests published even in recent years, although a number of tests have been conducted, classified as confidential, and submitted to the Agency to support pesticide registration. Those that have been published have been written by researchers familiar with bird tests and many details of protocol have not been included in the reports. ASTM (1979a) has prepared a draft avian reproduction method. Although this ASTM protocol is similar to the Office of Pesticide Programs protocol (US EPA 1978a), it is designed for a variety of species. Thus, the history

of avian reproduction test methodology is basically a history of Patuxent and EPA methods. This guideline continues the trend because these methods appear to be the most appropriate for developing data needed to make regulatory decisions and because no other methods have become accepted widely.

Even though the basic method is similar for most investigators, there have been a few points of difference or controversy. Yet there are very few data to address these differences and little ongoing research that might resolve conflicts. To promote uniformity and comparability of tests, some recommendations and requirements are standardized in this test guideline. Where such recommendations and requirements are controversial and are not sufficiently addressed by published data, it is hoped that research will be stimulated to resolve questions. If feasible, conditions and procedures that approximate natural conditions have been selected in preference to other options. Controversial points are addressed under Issues in this support document.

The use of avian reproduction tests in the assessment of chemical impacts on the environment is based on several factors. First, birds are an obvious and important component of the environment. Congress has indicated repeatedly that birds are worthy of protection by passing such laws as the Lacey Act of 1900, Migratory Bird Treaty Act of 1918, Migratory Bird Conservation Act of 1929, Pittman-Robertson Act of 1937, Fish and Wildlife Act of 1956, Endangered Species Act of 1973, and others. The United States also has entered into treaties with Great

Britain and Canada (1916), Mexico (1937), Japan (1974), and Russia (1976) for the protection of migratory birds. The people of the United States also have indicated a desire to protect birds through their support of the Audubon Society, Nature Conservancy, and other environmental groups. Sportsmen's organizations support protection of birds, although their interests often focus heavily on game birds.

Second, birds have a definite economic importance. Federal and State Agencies spend large sums for the preservation and propagation of birds. Hunters and birdwatchers also spend substantial sums in pursuit of their pastimes. Less measurable, but of definite importance, is the substantial role of birds in insect control.

Birds have an important ecological role. Insectivorous birds consume huge quantities of insects and other invertebrates, many of which are considered pests. Small mammals and other vertebrates or plants are consumed by various birds, sometimes to the extent that birds have an important effect on populations. In turn, birds are consumed by birds of prey, mammals, and other vertebrates. Excretory products of birds provide nutrients for plankton and other microorganisms that in turn are food for larger organisms. Birds are important in pollination of some plants and in dispersal of others. Because of their mobility, the effects of birds are not restricted to specific locations.

Finally, birds are among the more sensitive terrestrial vertebrates. Because of their high metabolic rate, high body temperature, and the demands of flight, they require more energy relative to their size than most other animals. The energy requirements lead to greater food

intake and thus to greater toxicant intake when a toxicant is in or on their food. There are abundant data showing that some birds, particularly raptors, pelicans, and waterfowl, are very sensitive in their reproductive responses to toxic chemicals (e.g., Ratcliffe 1967, Anderson and Hickey 1972) and that some species have suffered from drastic population reductions apparently as a result. Avian reproduction is unique, and no surrogate is adequate to predict effects on eggshells, the primary mode of impairment for many chemicals such as DDE (Heath et al. 1969).

Avian reproduction tests are extremely valuable in assessing the potential population effects of exposure of birds to toxic chemicals. The route of intake simulates natural exposure to chemicals on or in the food. Most physiological effects can be assessed under laboratory conditions, although many behavioral effects, such as nest desertion, are difficult to study in the laboratory. A positive finding of impairment in the laboratory is highly predictive (qualitatively) in the field when exposure is the same. However, negative findings in the laboratory may not preclude adverse effects under field conditions. Thus, some extrapolation may be made from laboratory to field, but quantitative extrapolation is risky.

1. Issues

The avian reproduction test guideline and support document contain some controversial points. Data are insufficient or absent to support either side of most points. For other points, there may be data supporting each side. A number of controversial points have been selected as potential research projects. Issues are merely

identified below and are discussed in the appropriate sections of this document.

- Is productivity from tests run out of normal season sufficient to evaluate the potential for reproductive impairment?
- Should methods involving natural incubation of eggs be incorporated into tests?
- Should the current use of first year birds only be continued or should proven breeders be used? Would either age produce results suitable for comparison?
- What carriers, if any, should be used or allowed for incorporation of the test substance into the diet?
- Are commercial foods adequate, or is there too much variation and/or contamination of commercial foods?
- How often should treated diets be mixed? Is there an advantage in mixing diets with decreasing concentrations to simulate natural degradation of test substances?
- What is the optimum number of birds to be tested in order to attain statistically valid results and still be cost-effective? Can tests using pairs only of birds in pens be successfully conducted by a variety of testing facilities?
- Can more useful results be obtained by testing enough dose levels to use linear regression analysis?
- Should outdoor tests be allowed?
- Should medication be allowed as is needed to treat individual sick birds

- Are the typical values (Section 2.1.6) and test acceptability criteria (Section 2.1.9) for productivity and other egg parameters realistic for a wide diversity of testing facilities, or are they really only valid for a few?

B. Test Procedures

1. Test Substance Concentrations

Test substance concentrations in the diet will be specified in the test rule. These concentrations will be based upon the properties of the test substance, the lethal and no-effect levels, if known, or the amount of test substance known or likely to be found in the environment. Three concentration levels are specified because, for many test substances, three levels will allow for a dose-response regression analysis from which a no- effect level can be calculated. (See section 2.1.8 on statistics for further discussion).

2. Controls

Concurrent controls are required for every test to assure that any observed effects are a result of ingestion of the test substance and not to other factors. Such other factors may include environmental factors such as temperature, lighting, vapors, sensitive or stressed test birds, etc. If a diluent is used in mixing the diet, this diluent also is used in the untreated diets in the same concentration as it occurs in treated diets. In effect, this results in a diluent, but no completely negative, control. Diluent choices are based upon their lack of toxicity (e.g., water, completely evaporated acetone) and it is not considered necessary, therefore, to have an

additional negative control when a diluent control is used.

3. Test groups and numbers of animals

A minimum of 8 replicate pens with one drake and three hens or 20 replicate pens with one drake and one hen is required for each test concentration. The number of replicates is needed to achieve a test with statistical significance. There is enough variation in productivity of mallard hens that fewer replicates would be very insensitive to all but the most severe effects.

The recommended arrangement is for use of group pens containing three females each. This is based upon the rather aggressive behavior of courting males that could result in severe stress to the hen, if only one hen were present. In mallard reproduction tests, Heinz (1976a) obtained satisfactory reproduction with mallard pairs. He attributed his success primarily to having a nest box and flowing water (Heinz, personal communication). Heinz has pushed strongly for the adoption of his method. Testing of pairs only is a practice that should lead to statistically stronger tests and will provide a better indication of effects on individuals. However, it is offered only as an option in this guideline because it is unknown at this time if other researchers will be able to achieve good results. The alternative arrangement of at least 20 mallard pairs per level is suggested to accommodate those test facilities that believe they will have success or that wish to contribute to the development of methods. A warning is included in the guideline so that relatively inexperienced investigators should not expect unqualified success. The additional pens in pair testing will strengthen notably the statistical

analysis of the test. This alternative is included because it is statistically stronger than the more familiar method of group testing.

4. Duration of Test

The avian reproduction test lasts approximately 22 weeks. The initial part of the test is an exposure phase where birds are receiving treated diet. Exposure to treated diets begins with this phase and allows for the test substance to act upon the reproductive mechanisms of the body, and also for birds to accumulate residues of lipophilic and other test substances. The development of reproductive capacity actually begins months before egg laying starts (Wolfson 1964). Therefore, exposure to the test chemical should be well in advance of egg laying, if the test is to investigate reproductive effects in general.

The second phase, following lengthening of the photoperiod, directly brings the birds into readiness for egg laying. The duration of this phase is dependent upon the response of the test birds.

The third phase is the egg-laying portion of the test. This is to be a minimum of eight weeks. The duration of this phase is based upon two main factors. First, it is important to determine if egg laying is within normal levels or if it declines, or otherwise varies, over a period of time. This yields information on speed of action and has a role in using the test for assessment purposes. Second, the eight week period is needed to provide sufficient data for a strong statistical analysis.

A withdrawal period is optional when impairment has been detected. This period may provide data on recovery of

reproductive capacity that could be useful for assessment purposes.

5. Preparation for reproduction (photoperiod)

Because photoperiod is critical to reproduction, it should be controlled in indoor tests. Under natural conditions, photoperiod is lengthening gradually just prior to the reproductive season. The earlier mallard reproduction tests were conducted outdoors (Stickel and Heath 1965, Heath et al. 1969, 1972b, Heath and Spann 1973), and therefore, used natural photoperiod. More recently, mallard reproduction tests submitted for pesticide registration purposes have been conducted indoors and egg laying has been induced by increasing photoperiod to 16 or 17 hours of light per day. The ASTM (1979a) and US EPA (1978a) both recommended seven hours of light in the first phase and 16-17 hours light in the second phase. Without giving supporting data, both of these protocols stated that the regime is for maximum egg production, and both specify at least an option of gradually increasing the length after egg laying has started. The photoperiod in this guideline is based on the above data and a lack of any suggested alternatives.

The dark period in the photoperiod should not be interrupted, even briefly, except as absolutely necessary. Kirkpatrick (1955) found that even as little as 15 minutes interruption on a short day/long night regime caused an increase in gonadal development of bobwhite, and all birds exposed to a 60 minute interruption became fully active sexually. It is highly probable, on the basis of Kirkpatrick's (1955, 1959) data, that it is the length of

the dark period rather than the light period that controls bobwhite reproductive preparation. It is likely that this holds for mallards also, although no definitive data were found.

Kirkpatrick (1955) also tested light intensity as a factor in avian reproduction. Responses of bobwhite were very similar at intensities of 0.1 to 100 foot candles on a 17 hour light photoperiod. With shorter days, the 0.1 foot candle intensity did not achieve the same results as 1,10, and 100 foot candles. These data support the approximate 6 foot candle intensity for bobwhite that was suggested by ASTM (1979a) and US EPA (1978a). In the absence of other data, the same light intensity has been specified for mallards.

6. Observations and Measurements

a. Adult Birds

Observations of food consumption, body weights, and signs of toxicity are required. Body weights are required three times during the test. More frequent body weights might be informative, but the stress of handling may offset the collection of data, particularly for laying hens. Food consumption is to be estimated at frequent intervals. This will provide data both on test substance ingestion and on energy intake for test birds. The latter data will be particularly helpful in the absence of frequent weighings.

Clinical signs of toxicity contribute substantially to the analysis of the data, in addition to providing information on the mode and speed of action. If dose levels are finely tuned, there should be minimal observed acute toxicity. However, without adequate preliminary data, one

or more of the test levels may cause lethal or notable sublethal effects. Such effects may affect the results of the test without directly acting on the specific reproductive parameters being investigated. For example, a severely stressed bird may not be able to mobilize internal resources to produce eggs. Signs of toxicity will give valuable information in evaluating the results of the test.

b. Duckling

Mallard eggs have been incubated in reproduction tests for as long as 27 days (Heath and Spann 1973). Greenwood (1975) reported average incubation lengths of 24.6 days for a wild mallard strain and 25.5 days for a game farm strain. US EPA (1978a) stated that eggs should be removed from hatchers on day 27. The meager data available suggest that 27 days is sufficient and this incubation length has been selected for this guideline.

Because the pen is the basic unit for statistical analysis, it is necessary that the eggs and hatchlings be identified as to pen of origin. This can be done either by housing all eggs or ducklings from one pen together, or by individually marking each egg and bird.

Environmental conditions for young ducklings have not been reported in available reproduction papers (Heath et al. 1969, 1972b, Heath and Spann 1973), nor have the US EPA (1978a) or ASTM (1979a) reproduction protocols suggested any housing conditions for ducklings. However, ASTM (1979b) and US EPA (1978b) dietary test protocols both have recommended procedures for housing young ducklings. Parameters given in these references include temperature of 35°C with a lower temperature outside the cage to provide a gradient,

galvanized brooder cages maintained indoors, and a commercial duck starter ration. These conditions have been included in this guideline.

Observations on ducklings are necessary to determine if and when toxicity might be expressed in the offspring. Although most test substances exert their action prior to hatching, some may affect growth, development, or survival of ducklings.

c. Eggshell Thickness

The classic example of reproductive impairment in birds is the eggshell thinning effect of DDE, a metabolite or degradate of DDT (Tucker and Leitzke 1979). It is important to measure eggshell thickness because as little as 11% thinning can have an effect on avian populations (Tucker and Leitzke 1979). Techniques given in this guideline are widely used (Heath and Spann 1973, ASTM 1979a, US EPA 1978a), although many papers do not give a full description of the process. It is particularly important for several measurements to be made around the girth of mallard eggs, in order to average out any aberrant single measurements that could affect results if only one or two measurements are taken.

d. Typical Observed Values

The typical observed values presented in this guideline have been taken from the Agency's pesticide guidelines (US EPA 1975, 1978a). A number of avian reproduction tests have been submitted to the Agency for the purpose of pesticide registration and have been classified as confidential. Most of the tests that have been conducted during the normal reproductive season have achieved for control birds the

typical values as presented in the test guideline. Heath and Spann (1973) and Heath et al. (1969, 1972b) achieved these values in control birds insofar as could be determined from their data.

The values presented in the guideline have been identified as an issue. It is known that some testing facilities routinely meet these norms. However, it is not known if the values are realistic when many testing facilities are considered.

7. Egg Collection, Storage, and Incubation

Egg storage prior to setting for incubation has been reported for mallards in only a few papers by one senior author and has been 16°C and 55% humidity (Heath et al. 1969, 1972b, Heath and Spann 1973). Stromberg (personal communication) stated that he stored bobwhite eggs at 16° and at as high a humidity as could be obtained from evaporating water; this was up to 80%, but at least 50% relative humidity. He also stated that turning the eggs daily was important to keep part of the shell and egg from excessive drying. US EPA (1978a) recommends 16°C and 65% relative humidity. This guideline specifies 16°C, but only a range of relative humidity between 55% and 80%, since many testing facilities do not have the means to control storage humidity and since this range has produced good viability.

Several papers were found that reported incubation temperatures and humidities for mallards. Temperatures were consistently 37.4-37.5°C and humidities ranged from 57-80% (Heinz 1976a, 1976b, Holmes et al. 1978, Greenwood 1975). Neither ASTM (1979a) nor US EPA (1978a) specify incubation temperatures although the latter does recommend 39°C and 70%

relative humidity during the hatching phase from day 23 to day 27. Stromberg (personal communication) stated that 99.75°F (37.6°C) and 50-70% relative humidity are standard commercial poultry incubation temperatures and that all testing facilities with which he is acquainted use commercial poultry incubators. A temperature of 37.5°C and relative humidity of approximately 70% have been specified in this mallard test guideline.

Candling procedures are a standard practice for determining eggshell cracks, fertility, and embryo survival. These procedures have been used in most mallard tests, but timing has been inadequately reported. US EPA (1978a) specifies, for mallard, candling on day 0 for cracks, on day 14 for fertility and on day 21 for embryo survival. This timing has been adopted in this guideline because it will permit comparisons of test data with existing studies in Agency files. At least one major avian testing laboratory protocol uses the same timing.

US EPA (1978a) recommended moving eggs to a separate hatcher or incubator for hatching, but gave no reasons. None of the available published papers mentioned this procedure. However, Stromberg (personal communication) stated that this procedure is highly recommended as a means to minimize disease vectors. This procedure has been recommended in the guideline.

This test guideline does not consider the effects on incubation behavior, nest desertion, and care of young. Techniques for investigating the effects of chemicals on these behaviors are in their infancy and, at present, are prohibitively expensive. Agency research has been proposed

to investigate methods of incorporating natural incubation into future reproduction guidelines at a reasonable cost.

8. Required Analyses

a. Statistical

The statistical analysis of avian reproduction studies typically has been analysis of variance (Heath and Spann 1973, Heinz 1976a, US EPA, 1978a, ASTM 1979a) using the parameters set forth in this guideline. Angular transformations have been used to a lesser extent (Heath et al. 1972b). Regression analysis is a powerful tool that may be used if there are data at sufficient dose levels to obtain a dose-response curve. Regression analysis has been recommended, as an option by ASTM (1979a), and methods directed toward regression analysis are being developed (Stromberg, personal communication). It is the opinion of the author of this test guideline that regression analysis is a more useful tool than currently typical methods because it yields a dose response curve and this curve can be used for extrapolation. Analysis of variance provides only significance at a particular level and does not lend itself to extrapolation on a reliable basis. At the present time, methods and background work have not been developed for a test oriented primarily towards a dose-response curve. More dose levels would be needed, possibly with fewer animals per level. This subject may be included in the Agency's research projects. In the meantime, this guideline uses methods appropriate to analysis of variance.

b. Test Substance Concentrations

Samples of treated diets will be analyzed to determine the actual levels to be used in the test. Analysis will

help to detect mathematical errors in calculating concentrations, technicians' errors in mixing diets, and manufacturers' errors in determining the amount of active ingredient in a test substance (Heinz, personal communication). All test substance concentrations will be analyzed so that, even with only three test concentrations, dose response curve (if obtained) would be based on measured concentrations, rather than on nominal levels. A second analysis about midway through the test will corroborate initial levels.

c. Basal Diet

Most testing facilities use diets prepared by commercial feed companies. Some facilities may have a commercial company prepare a diet to order. Normally, such diets are supplied with a quantitative list of ingredients, and such a list should be supplied with the test report. If there are supplements added to the diet, a list of all supplemental ingredients also should be submitted. Analysis of ingredients in the basal diet is important because there are a number of potential test substances, such as certain metals, that may interact with components of the diet and possibly affect the results of a test. A nutrient analysis will allow for a better evaluation of such results. In addition, it is possible that dietary deficiencies or imbalance of ratios of nutrients also could affect the results. Even though commercial companies normally supply a nutritionally adequate diet, it is important to know the components because no rigid requirements exist for the type and constitution of the diet used.

9. Acceptability Criteria

Test acceptability is dependent upon following the mandatory requirements and achieving acceptable control productivity and survival. When control values do not reach the typical observed values as discussed in Section 2.3.6, it is very likely that there are problems with some aspect of the test. Since reproductive parameters of treated birds are compared with controls, it is essential that values for control birds are normal as determined from similar reproduction studies.

Rather than requiring every parameter to achieve normal values, it was decided that test acceptability should be based on the final parameter of 14-day old survivors per control hen as one criterion. For mallards, the requirement of 14 survivors per hen over a ten week period was selected because data in the Agency's pesticide files show that acceptable tests have achieved and usually surpassed this level. Heath et al. (1969) reported 16.1 and 16.4 control survivors per hen for eight week seasons. Heath and Spann (1973) reported 6.1 and 6.2 control survivors per hen for four week seasons, which values are equivalent to 15 survivors per hen for a ten week season. Heinz (1979) achieved an annual average of 46 7-day survivors per control hen for 3 years, using proven breeders and a season of unknown length.

Eggshell thickness of control birds is also a criterion of test acceptability because thin eggshells among control birds are usually a sign of inadequate diet, which in turn could affect other aspects of the test. A number of mallard reproduction tests have had average control eggshell

thickness of .365 to .393 mm (Heath et al. 1969, 1972b, Heath and Spann 1973, Heinz 1979). Heath and Spann (1973) also reported individual extremes of .330 and .420 mm. Holmes, et al. (1978) reported .343 and .363 mm average thickness for unfertilized eggs from mated control females. The Agency's pesticide guidelines (US EPA 1978a) state that normal eggshell thickness for mallards is .31 - .33 mm. On the basis of the other published data cited above and additional reproduction studies in the Agency's pesticide files, it appears that .31-.33 mm is unrealistically low. The normal values given in section (h)(11) of the test guideline are .35-.39 mm, however, at the present time, it seems appropriate to lower the acceptable level to .34 mm. This may be revised pending receipt of additional data and public comment.

A well conducted test with adult birds should not result in any but an occasional mortality in control groups, even though the test is relatively long. This is especially true since this guideline provides for rearrangement of incompatible birds during the acclimation period. Therefore, control mortality in excess of 10% indicates problems with some aspect of the test.

C. Test Conditions

1. Test Species

a. Selection

The mallard, Anas platyrhynchos L., is the species to be tested. The choice of mallard is based on a number of factors. The mallard has a widespread distribution, not only in the United States, but also in Eurasia. Such distribution means that mallards may be exposed to toxic

substances in the environment regardless of the location of the toxic substance. As waterfowl, mallards may also be exposed to toxic substances in the water, in sediments, and on land. Because the mallard is the most abundant and widely distributed duck in the northern hemisphere (Bellrose 1976), it is also suitable as a native test species for many countries belonging to the Organization for Economic Cooperation and Development (OECD).

Mallards have been one of the more frequently used native bird species in reproduction tests with toxic chemicals, but there are still only a few published experimental studies with the species. DeWitt et al. (1963) found that mallards were more susceptible to reproductive impairment from several chemicals than were bobwhite or pheasant. Heath et al. (1969) showed that mallards are susceptible to eggshell thinning effects from DDE. The Agency's confidential pesticide files contain additional data confirming mallard sensitivity to eggshell thinning and other reproductive impairment. When reproductive tests are conducted on both gallinaceous birds and waterfowl, this combination of species will often indicate impairment that might not be found if only one species were tested.

The mallard is also amenable to testing in the laboratory. Mallards can be bred in captivity and are readily available from commercial sources so that testing of this species will not deplete wild stocks. There is sufficient information on the nutritional, habitat, and behavioral characteristics of natural populations of mallards in order to meet the basic nutritional and physical requirements of the species in the laboratory.

An advantage of reproduction testing with mallards or bobwhite instead of other birds is that mallard and bobwhite are typically the two species used in short-term toxicity tests with birds. There is an advantage in using the same species in reproductive tests that also can be used in short-term or field tests. Mallards have been used for short-term laboratory tests and actual or simulated field tests from the beginnings of ecological effects testing (DeWitt 1956) to the present (US EPA 1980b). Thus, the choice of mallard as a test species facilitates comparisons of the results from different kinds of tests.

In addition, mallards are generally considered to have a positive economic value. Although the Agency is charged with the protection of all species in the environment, the choice of an economically valuable species for testing is appropriate to the cost-benefit or risk-benefit analyses upon which Agency decisions frequently are based. Finally, there is as good a comparative data base for reproduction tests with mallards as for any native bird.

If a test is to simulate toxicity to naturally occurring populations of mallards, then it is important to use birds that are phenotypically indistinguishable from wild birds. Since many chemicals act upon specific enzymes, and enzymes are based on the genetic code, the use of birds genotypically similar to wild birds would be desirable. However, the determination of phenotype is a simple observational process, whereas genotypic determination is impractical, if possible at all. In addition, wild birds have a degree of heterogeneity that would not be typical of any given genotype.

The necessity for using healthy birds is obvious since the test is designed to determine toxicity to typical populations. It is admitted that not all birds occurring in natural populations are healthy, but the majority of survivors in natural environments are healthy. The health of birds is also important in reducing the number of variables that limit comparisons between tests. There are several checks in this test guideline that help to ensure that healthy birds are used. The use of previously untested birds not selected for resistance and being from disease-free flocks provides a basically healthy stock. Visual observations select out abnormal or unhealthy birds from that stock. A final check on health is based on the birds ability to survive two weeks immediately preceding exposure. The 3% maximum mortality during this period allows for an occasional death that may occur during acclimation and the time when unfamiliar birds in a pen are becoming adapted to each other.

The choice of first year birds was made in this guideline primarily because it facilitates comparisons with existing data and data that may be generated according to the US EPA (1978a) protocol for mallard. It also ensures that birds will be the same age; whereas if no age were specified, birds in the same test could be various ages. Use of first year birds also reduces the cost of birds and ensures that test birds will not have been used in previous reproduction tests.

2. Maintenance of Test Species

a. Acclimation

An acclimation period is necessary for birds to become familiar with the test environment. Ideally, birds will be maintained in test cages for several months, but this is impractical for testing facilities that purchase adult birds. It is also sometimes necessary to alter the composition of birds in a pen because of excessive aggression or other incompatibilities. The acclimation period allows time for rearranging incompitible birds.

b. Diet

There are few data on the detailed nutritional requirements for mallards. This subject is being investigated under a current contract and is a proposed research need. At the present time, a commercial duck breeder ration is recommended. All known testing facilities in this country use a commercial ration or a similar but specially prepared ration made by a commercial company. There are no known data to support an alternative diet for mallards. Changes may be made in the future when additional data become available. The recommendation of a commercial duck starter ration is made for the same reasons.

Samples of treated diets will be analyzed to determine the actual test concentrations used in the test.

3. Facilities

Only a few mallard reproduction tests have been reported in the literature, although a number have been submitted to the Agency and classified as confidential. As a result, it is difficult to define optimal conditions and most conditions have been recommended, rather than required.

Heath et al. (1969, 1972b) and Heath and Spann (1973) used outdoor pens with concrete floors for their mallard reproduction studies. Heath and Spann (1973) specified the size of pens as 10 X 20 feet; they housed 5 hens and 2 drakes in each pen. Heath, et al. (1969, 1972b) used the same pens and housed 5 or 6 hens and 2 drakes in each pen. Heinz (1976b) tested one drake and four hens in 5 x 10 ft. pens, but in another study tested mallard pairs in one meter square (10.7 square feet) pens (Heinz 1976a). Further housing details were not mentioned.

US EPA (1978a, 1980b) and ASTM (1979a) have both developed protocols for avian reproduction tests. Rationales for selecting particular conditions were not spelled out in either protocol. Reasons were apparently based on experience with reproduction tests that have been submitted to the Agency for pesticide registration and that have been classified as confidential. These three protocols have specified size only as being "adequate". All three protocols recommend 21°C, 55% relative humidity, and adequate ventilation. The same conditions have been recommended in this guideline, since these protocols are the most widely used and data developed from this guideline will be comparable with data gathered using all three protocols.

Galvanized metal is the recommended construction material. This material has been used widely in toxicity tests (Heath et al. 1972a, Hill et al. 1975) and has been recommended in ASTM (1979a) and US EPA (1978a) protocols for reproduction tests. The ASTM (1979a) protocol suggests stainless steel or perfluorocarbon plastics (e.g., Teflon) as alternatives. As a relatively non-adsorbent material,

perfluorocarbon plastics may be used to coat wood or other materials that might be contaminated by chemicals if used uncoated. Because of the tendency of wood to sorb chemicals, uncoated wood may be not be used where it is likely to become contaminated and come in contact with birds in subsequent tests.

Pens should be cleaned and sanitized between tests. Brushing and/or steam cleaning appear to be the most appropriate since they do not involve the use of chemicals that could affect subsequent tests. Detergents and bleach have been used by Denver Wildlife Research Center (Tucker, personal communication) and Patuxent Wildlife Research Center (Heinz, personal communication). The use of chemical disinfectants, such as quaternary ammonium compounds, should be avoided because of possibility that these compounds can leave toxic residues. However, the widely used cold sterilization method with ethylene oxide is acceptable, if needed for disease control. Pens should not be cleaned during a test in order to minimize disturbance to the test birds.

4. Environmental Conditions

a. Temperature (See Section II.C.3)

b. Humidity (See Section II.C.3)

D. Reporting

The information that is required to be reported in section II.B.8.b is essential to a proper evaluation of the test reults. These required items are needed (1) to establish that the test was conducted according to specifications, (2) to evaluate those conditions and procedures that could affect the results of the test, and (3) to supply the Agency with sufficient information to

conduct an independent analysis of statistics and conclusions. The location of the raw data storage will allow the Agency to find additional information that may have been left out of the report or that may be needed for enforcement purposes. The location is necessary because some chemical companies request the testing facility to keep these data, while other companies keep their own. The information is needed in a detailed manner because this avian reproduction guideline contains few rigid requirements. Even when minimums or maximums are specified, it is important to know how much the test may have exceeded specifications, such as; if test birds were observed more frequently than required, if the number of test concentrations exceeded the three levels required, etc.

The information required in section II.B.8.b on Test Substance Concentration to be available, but not included in the test report, may be needed if there are serious concerns about the results or validity of the test. This information will not normally be needed and therefore is not required routinely.

III. Economic Aspects

Three laboratories were surveyed to estimate costs for performing the test outlined in this guideline. The individual laboratories gave prices of $22,000, $12,650, and $40,000. The "best estimate" based upon the survey was $24,883. A cost estimate also was made by separating the protocol into components and estimating the cost of each component, including direct labor cost, overhead cost, other direct cost, general and administrative cost, and fee. The best estimated final cost, based upon this calculation

method, was $24,129, with an estimated range of $12,064 to $36,193 based on ± 50% of the best estimate. The calculated estimate is similar to the best estimate based on the survey. Marked differences in prices obtained from the different laboratories may have resulted from a number of factors, such as nature of the chemical, overhead rates, outside consultants, automation, marketing strategies, and other factors as outlined in a cost analysis report by Enviro Control (1980). The cost estimates were made assuming that the requirements of the Good Laboratory Practice Standards, as specified in section (d) of the Mallard reproduction guideline, are being satisfied.

In a cost analysis of subpart E pesticides guidelines, laboratories were surveyed in 1978 and in 1980 to determine the cost of testing (US EPA 1980a). The cited costs did not differentiate between species, however, the unit cost for an avian reproduction test was $24,000 in 1978 and $28,000 in 1980.

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EG-18, OECD
August, 1982

DAPHNID CHRONIC TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

DAPHNID CHRONIC TOXICITY TEST

(a) Purpose. The proposed Daphnia chronic toxicity test standard is designed to assess the effects of test substances on the survival and reproduction of Daphnia as a representative freshwater invertebrate. The duration of the test permits the daphnids to be exposed to a chemical from shortly after birth until well into adulthood. The organisms are exposed long enough to allow the adults to produce several broods of progeny. Initiating exposure shortly after birth allows an assessment of the possible effects of the test chemical on such processes as reproduction, maturation, fecundity and growth.

(b) Definitions. The following definitions apply to this standard:

(1) "Acute lethal toxicity" is the lethal effect produced on an organism within a short period of time of exposure to a chemical.

(2) "Confidence limits" are the limits within which, at some specified level of probability, the true value of a result lies.

(3) "LC50" is the median lethal concentration, i.e. that concentration of a chemical in air or water killing 50

percent of a test batch of organisms within a particular period of exposure (which shall be stated).

(4) "Reference substance" is a chemical used to assess the constancy of response of a given species of test organisms to that chemical, usually by use of the acute LC50. (It is assumed that any change in sensitivity to the reference substance will indicate the existence of some similar change in degree of sensitivity to other chemicals whose toxicity is to be determined).

(5) "Static test" is a toxicity test with aquatic organisms in which no flow of test solution occurs. Solutions may remain unchanged throughout the duration of the test.

(6) "Renewal test" is a test without continuous flow of solution, but with occasional renewal of test solutions after prolonged periods, e.g., 24 hours.

(7) "Flow-through test" is a toxicity test in which water is renewed continuously in the test chambers, the test chemical being transported with the water used to renew the test medium.

(8) "Time-response curve" is the curve relating cumulative percentage response of a test batch of organisms, exposed to a single dose or single concentration of a chemical, to a period of exposure.

(9) "Toxicity curve" is the curve produced from toxicity tests when LC50 values are plotted against duration of exposure. (This term is also used in aquatic toxicology, but in a less precise sense, to describe the curve produced when the median period of survival is plotted against test concentrations).

(10) "Units" all concentrations are given in weight per volume (e.g., in mg/liter).

(c) Test procedures--(1) Summary of the test. (A) Test chambers are filled with appropriate volumes of dilution water. In the flow-through test the flow of dilution water through each chamber is then adjusted to the rate desired. The test substance is introduced into each test chamber. The addition of test substance in the flow-through system is done at a rate which is sufficient to establish and maintain the desired concentration of test substance in the test chamber.

(B) For the renewal test, the test is started within 30 minutes after the test substance has been added and uniformly distributed in the test chambers. In the flow-through test the test begins after the concentration of test substance in each test chamber of the flow-through test system reaches the prescribed level and remains stable. At

the initiation of the test, daphnids which have been cultured or acclimated in accordance with the test design, are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically during the test, immobile adults and offspring produced are counted and removed, and the findings are recorded. Dissolved oxygen concentration, pH, temperature, the concentration of test substance, and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to determine any significant differences ($P \leq 0.05$) in immobilization and reproduction as compared to the control.

(2) [Reserved]

(3) Range-finding test. (i) General A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) Introductory information for range-finding test.

(A) Prerequisites:

(1) Water solubility.

(2) Vapor pressure.

(B) Guidance information:

(1) Structural formula.

(2) Purity of the substance.

(3) Methods of analysis for the quantification of the substance in water.

(4) Chemical stability in water and light.

(5) n-octanol/water partition coefficient.

(6) Results of a test on biodegradability.

(C) Qualifying statement. For chemicals with low solubility under test conditions, it may not be possible to quantitatively determine the EC50.

(iii) Methods for range-finding test. (A) Definitions and units:

(1) 24 hour EC50. The concentration (based upon nominal concentration) calculated to have immobilized 50 percent of the daphnids by 24 hours exposure. (If another definition is used, this shall be reported, together with its reference).

(2) Immobilization. Those animals not able to swim for 15 seconds after gentle agitation of the test container are considered to be immobile. (If another definition is used, this shall be reported, together with its reference).

(B) Reference substances. In the course of the acute immobilization phase a reference substance may occasionally be tested for EC50 with the test compound as a means of assuring that the laboratory test conditions are adequate

and have not changed significantly. An example of such a useful reference substance is $K_2Cr_2O_7$.

(C) Conditions for the validity of the range-finding test:

(1) The mortality in the controls should not exceed 10 percent at the end of the test.

(2) The oxygen concentration at the end of the test shall be > 70 percent of the air saturation value at the temperature used.

(3) Test Daphnia should not have been trapped at the surface of the water, at least in the control.

(4) If conducted, the results with the reference compound should be within the normal range for the laboratory conducting the test.

(5) If the EC50 is not calculable due to an inadequate number of intermediate response levels, it is acceptable to merely report the highest concentra-tion causing complete immobility, provided that the concentration factor between doses was $\leq$ 1.8.

(D) Performance of the range-finding test:

(1) Equipment which will come into contact with the test solutions should be glass. This glassware should be cleaned with solvents known to remove previously tested chemicals.

(2) Any water, either reconstituted or natural water, can be used, provided that it will sustain growth and reproduction of Daphnia without signs of stress.

(3) At least 20 animals should be used at each test concentration, preferably divided into four batches of five animals each.

(4) At least 2 ml of test solution should be provided for each animal.

(5) The test temperature should be between 18 and 22°C, and for each test it should be constant within ± 0.5°C.

(6) A light-dark cycle is optional.

(7) The concentrations should be formulated in a geometric series, preferably without using any solvents. If solvents, solubilizing agents, emulsifiers, etc., have to be used, they should be commonly used adjuvants and not be toxic in themselves at the levels used. Neither should they have a synergistic or antagonistic effect on the toxicity of the substance tested. In no case should the concentration of an organic solvent exceed 0.1 ml/l.

(8) The test solution should be prepared before intrododuction of the daphnids.

(9) The test solutions should not be aerated.

(10) The daphnids shall not be fed during the test.

(11) The highest concentration to be tested should not exceed 1.0 g/l.

(12) Concentrations sufficient to lead to zero and 100 percent immobilization and the 24 hour EC50 should be tested together with a control.

(13) The pH and the oxygen concentration of the blank and all the test concentrations should be measured at the beginning and the end of the test. The pH of the test solutions should not be modified.

(14) Volatile compounds should be tested in completely filled, closed containers, large enough to prevent lack of oxygen.

(4) Definitive test. (i) General. The results of the range-finding test are used to determine, with judgement, the concentration levels to be used in the definitive test. It is suggested that this reproduction test be carried out using a geometrical concentration series of at least five concentrations with an interval of at least 10, starting at approximately the 24 hour EC50 concentration and ending at 1/100 of the 24 hour EC50. If necessary, lower concentrations are to be tested.

(ii) Introductory information for the definitive test. (A) Prerequisites:

(1) Water solubility.

(2) Vapor pressure.

(3) Chemical stability in water and light.

(4) Results of a test on biodegradability.

(5) 24 hour EC50 or the highest concentration producing no immobility and the lowest concentration causing complete immobility.

(B) Guidance information:

(1) Structural formula.

(2) Purity of the substance.

(3) n-octanol/water partition coefficient.

(C) Recommendations. (1) Instead of a two week test in which three batches of young should be born per female, a test of three or four weeks may be preferred in order to obtain a more thorough judgement of the influence of the test substance on mortality and reproduction. In this period approximately six to nine batches of young should be born per female.

(2) It is recommended that a statistical test (such as an analysis of variance) be used to determine whether the test replications can be analyzed together.

(iii) Criteria for a valid definitive test. (A) Control mortality should not exceed 20 percent at the end of the test.

(B) The oxygen concentration shall have been > 70 percent of the air saturation value throughout the test.

(C) The pH for the controls and for at least the most concentrated solutions shall be known throughout the test. The deviation from the initial value at the beginning of the test should be $\leq$ 0.3 units.

(D) The first young should have been born in the controls after a maximum of nine days.

(E) The average cumulative number of young per female in the controls after three broods, should be > 20 at a temperature of 20° $\pm$ 0.5°C.

(iv) Definitive test procedures. (A) At least 40 animals should be used at each test concentration, preferably divided into four batches of ten animals each. The test concentrations are made up in a geometric series, and if possible, without any solvents.

(B) Every test shall include controls consisting of the same dilution water, conditions, procedures and daphnids from the same population (culture container), except that none of the chemical is added.

(C) The minimum duration of the test is 14 days, in which at least three broods of the F1 generation shall have appeared in the controls. If this is not the case, the test shall be continued until the third brood in the control is complete. If desired, the test can be continued for a total period of three to four weeks, even if three broods are born within three weeks.

(D) The live and dead daphnids of the "parental" generation (P) are counted and the dead specimens removed. This should preferably be carried out daily, but at least every two days, e.g. Monday, Wednesday and Friday.

(E) The presence of eggs in the brood pouch, males or epphipia shall be recorded. The condition and size of the parent generation should be visually compared with the controls.

(F) When the parental animals are about seven days old, the first young daphnids emerge from the brood pouch. After this, a new batch appears every two to three days. These batches are called "broods" of the F1 generation.

(G) The newborn young of the F1 generation are counted at least every two days (Monday, Wednesday and Friday) and their estimated condition (based on visual examination) is

recorded. After counting and examination, the young are poured away. The presence of eggs from which no young have emerged (on the bottom of the test vessel) is recorded.

(5) [Reserved]

(6) Analytical measurements. (i) Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample shall be validated before beginning the test by appropriate laboratory practices. An analytical method should not be used if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) Samples of the test substance should be taken at the beginning and during the test. The actual concentration shall not drop below 80 percent of the nominal concentration. Aeration of the test solutions is permissible, unless this would cause the actual concentration of the test substance to drop below 80 percent of the nominal concentration.

(iii) The oxygen concentration in all test solutions shall be checked once every 48 hours (if desired, every Monday, Wednesday and Friday).

(iv) The pH of the controls and of at least the most concentrated solution shall be checked before and after each renewal in the renewal test and once every 48 hours in a flow-through test.

(d) Test conditions--(1) Test species--(i) Selection. Daphnia magna less than 24 hours old at the beginning of the test, laboratory bred, free from known diseases and with a known history (breeding method, pretreatment) are used in this test. Other Daphnia species may be used provided that the relevant re-production parameters are comparable to those of Daphnia magna.

(ii) Feeding. The daphnids should be fed at least daily during the definitive test. In the chronic daphnid test, food (in any quantity) of any kind that meets the criteria of reproduction for validity of the test, is acceptable. Overloading of the test system with food should be avoided in order to minimize sorption of the test substance. Log-phase, unicellular green algae are generally suitable.

(2) Facilities--(i) Apparatus. (A) Normal laboratory apparatus and equipment should be used. Equipment which will come into contact with the test solutions should preferably be all glass. This glassware should be cleaned with solvents known to remove previously tested chemicals.

(B) This reproduction test should not be carried out in a static test system; either a renewal or flow-through system shall be used. The renewal period should be guided by the chemical analysis and (if applicable) the oxygen level in the test solution. The solutions shall be renewed at least once every 48 hours (if desired, on Monday, Wednesday and Friday).

(C) Volatile compounds should be tested in completely filled closed containers, large enough to prevent the oxygen concentrations from falling below 70 percent of the saturation value. An almost-closed, flow-through system may also be used. When more than 20 percent of the test compound would be lost through volatility, the test should be carried out either in a flow-through system or in an enclosed container of sufficient size to ensure that the oxygen level does not fall below 70 pecent of the saturation value.

(ii) Cleaning. If the renewal scheme is used, the glassware shall be emptied and food residues removed at renewal. It is recommended that the glassware be rinsed with deionized water and kept as a coded series for the following renewal. Each test unit therefore has two vessels which are used alternately. If flow-through systems are used, these should be cleaned twice a week.

(iii) Dilution water. Surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if daphnids will survive and reproduce in it for the duration of the culturing, acclimation, and testing periods without showing signs of stress. The quality of the diluton water should be constant and should meet the following specifications:

Substance	Maximum Concentration
Particulate matter	20 mg/l
Total organic carbon or	2 mg/l
chemical oxygen demand	5 mg/l
Un-ionized ammonia	20 ug/l
Residual chlorine	1 ug/l
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs)	50 ng/l
or organic chlorine	25 ng/l

(3) Test parameters--(i) Carriers. If solvents, solubilizing agents, emulsifiers, etc., have to be used, they should be commonly used adjuvants and should not themselves be toxic at the concentrations used. They should also not interact to alter the toxicity of the substance under test. In no case should the concentration of an organic solvent exceed 0.1 ml/l.

(ii) Dissolved oxygen. The oxygen concentration shall be > 70 percent of the air saturation value throughout the test.

(iii) Lighting. A light-dark cycle is necessary for the definitive test; 8 hours darkness and 16 hours light are recommended.

(iv) Loading. At least 40 ml of test solution should be provided for each animal in the definitive test.

(v) Temperature. The test temperature should be between 18 and 22°C, but for each test it should be constant, within $\pm$ 0.5°C.

(e) Reporting (i) Test substance information:

(A) chemical designation.

(B) additional designations, e.g. trade name.

(C) empirical formula.

(D) manufacturer.

(E) batch number.

(F) degree of purity.

(G) date of sampling.

(H) water solubility.

(I) vapor pressure.

(J) biodegradability.

(K) chemical stability in water and daylight.

(L) n-octanol/water partition coefficient.

(ii) General information:

(A) Source of Daphnia, any pretreatment, breeding method (including source, kind and amount of food, feeding frequency), species identification and method of verification.

(B) Name and address of the testing laboratory, name of the person responsible for carrying out the test (study director).

(C) Name and address of sponsor.

(D) Dates of testing.

(E) Description of the test method or reference to the method used.

(iii) Conditions of testing:

(A) Carriers and/or additives used and their concentrations. If it is observed that the stability or

homogeneity of the test substance cannot be maintained, then care should be taken in the interpretation of the results and note made that these may not be reproducible.

(B) Dilution water: source and chemical and physical characteristics including at least hardness, pH, Ca/Mg ratio, Na/K ratio, alkalinity.

(C) Test temperature.

(D) Light quality, intensity and periodicity.

(E) All measurements of pH and oxygen level made during the test, preferably in tabular form.

(F) Results and date of test performed with reference compound if available.

(G) Description of test vessels: volume of solution, number of test organisms per vessel, number of test vessels per concentration, conditioning of the test vessels, the introduction of the test substance in the dilution water.

(H) In case of renewal, the renewal procedure and scheme. In case of flow-through, the test substance delivery system, the flowrate, periodicity of cleaning and technique used.

(I) If measured, the actual concentrations of the test substance and the dates of measurement.

(J) Number and percentage of daphnids that showed any

adverse effect in the controls and in each treatment at each observation period and a description of the nature of the effects observed, e.g. immobilization, mortality in tabular form.

(K) Description or reference to statistical procedures applied.

(L) Any other effects differentiating organisms in tests and controls.

(iv) Specific range-finding and definitive test information: (A) For the 24 hour EC50 (acute immobilization) phase also report:

(1) The 24 hour EC50 perferably with 95 percent confidence limits, either by computation or graphically, and the method applied. The probit method is recommended.

(2) If possible, the slope of the concentration response curve with its 95 percent confidence limits.

(3) The highest tested concentration producing no immobile daphnids.

(4) The lowest tested concentration producing 100 percent immobile daphnids.

(5) Any other effect observed and the concentration at which it occurred.

(B) For the reproduction phase also report:

(1) The EC50 (immobilization) and LC50 values as far as possible at 24 hours, 48 hours, 96 hours, 7 days, 14 days and at the end of the test, preferably with 95 percent confidence limits, either by computation or graphically, and the method applied. For the determination, a probit method should be used.

(2) The length of time for the appearance of the first brood for each concentration

(3) The number of young alive in each test vessel on given days when counts were made (the minimum requirement is for counts at 48 hour intervals on Mondays, Wednesdays and Fridays).

(4) The number of dead young in each test vessel on given days when counts were made.

(5) Source, kind and amount of food; feeding frequency.

(6) If the recommended concentration scheme was followed and no effectss on reproduction are detected, then the results may be reported as being greater than the highest concentration tested.

(7) For each of the observed effects a statistical analysis of the homogeneity of replicate results for each concentration should be made. If homogeneity is found, it

should be determined through an appropriate statistical analysis, whether a significant difference exists between the control and the test concentrations.

(8) The highest concentration tested at which no significant difference is found compared to the controls with respect to mortality, reproduction and other observed effects.

(9) The lowest concentration tested with significant difference compared to the controls.

(10) Any other parameter can be reported at the option of the study director.

EG-19, OECD
August, 1982

ALGAL ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FRESHWATER ALGAE ACUTE TOXICITY TEST

(a) Purpose. (1) A unicellular green alga is used as a model system initially to estimate the concentration of a chemical which could affect the primary production of plants. Because regeneration times for unicellular algal species are measured in hours, this relatively short test can assess effects over several generations. Results allow the assessment of effects on initial organisms from short term exposures and give an indication of the effect on algal populations.

(2) Many different protocols for algal tests are available. This growth test is easy to perform and gives reproducible results with the recommended species. This Test Guideline can be adapted for other algal species. If such an adaptation is used, a description of the method should be provided with the test report.

(b) Definitions. The following definitions apply to this guideline:

(1) "EC-X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Growth rate" means an increase in biomass or cell numbers of algae per unit time.

(3) "Inhibition" means any decrease in the growth rate of the test algae compared to the control algae.

(4) "Limited water-soluble substances" means chemicals which are soluble in water at less than 1,000 mg/l.

(5) "Readily water-soluble substances" means chemicals which are soluble in water at a concentration equal to or greater than 1,000 mg/l.

(c) Test procedures--(1) Summary of the test. (A) The procedures for the preparation of the algal suspension, the stock solution of the test chemical, and the test media, are dependent on the solubility of the chemical and modifications in the testing procedure may be necessary due to the chemical's solubility in water. For chemicals with low solubility under test conditions, it may not be possible to quantitatively determine the EC-50.

(B) For purposes of the test, algae are grown in Erlenmeyer flasks in an environmentally controlled growth chamber. The test is started when 50 ml of algal suspension ($1x10^4$ or $2x10^4$ cells/ml) and 50 ml of the appropriate test chemical dilutions are placed in the flasks. Algal growth is measured at 24 hour intervals for at least 96 hours. A Coulter Counter, counting chamber, or other appropriate instruments may be used to determine cell density. The data

are used to define the concentration-response curve and the time-growth curve, and to calculate the EC-50 and the no-effect level (EC-0) for the chemical.

(2) [Reserved]

(3) Range-finding test. A range-finding test should be conducted to establish test chemical concentrations for the definitive test.

(4) Definitive test. (i) A definitive test is used to determine time-growth and concentration-response curves, as well as the EC-50 and no effect level (EC-0) of the test chemical. The testing method will vary slightly depending upon whether the chemical is readily water-soluble, of limited solubility, or volatile.

(ii) Criteria for a valid definitive test. (A) Algae in the control flasks should exhibit log phase growth within 48 hours of test initiation and should produce a standing crop of at least 10^5 cells/ml in 96 hours.

(B) At 96 hours, one test concentration should show no significant decrease in growth rate and one concentration should show greater than a 50 percent decrease in growth rate relative to the control.

(C) The pH of each test solution should be measured before use and, if necessary, adjusted to 7.5 $\pm$ 0.2 using HCl or NaOH.

(D) The test chemical concentration should be determined before and after the test when practical.

(E) No more than 20 percent of the test chemical should be lost by volatilization. If more is lost (or is likely to be), the test should be conducted in closed flasks with a resulting lower standing crop.

(iii) Test procedures dependent on solubility.--(A) Readily water-soluble chemicals. When readily water-soluble chemicals are tested, the following procedures are recommended:

(1) A stock solution of the readily water-soluble chemical should be prepared with micropore-filtered (0.45 um pore size) medium. The concentration of the stock solution should be twice as high as the highest concentration to be used in the test. From this stock solution at least five dilutions should be made. The dilutions should be in a geometric series with a ratio of 10 or n/10 (where n = number of dilutions). A minimum of five concentrations should be used such that the highest concentration results in at least 50 percent growth inhibition and the lowest concentration shows no significant difference (a = 0.05) from the control.

(2) The algal suspension for testing readily-soluble chemicals should have a density of approximately $2x10^4$ cells/ml of medium. The cell density should be quantified prior to beginning the test.

(3) The test should be carried out in triplicate (i.e. 3 replicate flasks per concentration). The test begins by transferring 50 ml of algal suspension to 250 ml Erlenmeyer flasks. Then, in sequence of increasing concentrations, a 50 ml volume of each prepared dilution is added to the appropriate flask. The control flasks receive 50 ml of medium. The flasks are then gently shaken and placed in the test chambers. The algal concentration in samples from each flask is determined at intervals of at least 24, 48, 72 and 96 hours after the start of the test and the number of cells or biomass (dry weight) per ml is calculated for each sample.

(4) A fluorimeter or spectrometer can be used to calculate cell number or biomass, but will not provide precise measurements at the start of the test and at 24 hours. If a Coulter Counter, or spectrophotometer, is used to enumerate algae at the beginning of the test, 100 ml of medium should be used to determine the background.

(B) Limited water-soluble chemicals. When chemicals with limited water-solubility are tested, the following modifications of the above procedure are recommended:

(1) A stock solution for chemicals with limited water-solubility should be prepared with a suitable organic solvent. This stock solution should be 10^4 times as concentrated as the highest concentration to be tested and the amount of solvent necessary to dissolve the chemical should not exceed 0.1 ml/l at the highest chemical concentration used.

(2) An algal suspension for testing chemicals with limited water-solubility should have a density of approximately 10^4 cells/ml. One hundred (100) ml of this algal suspension (10^4 cells/ml) are placed in each flask and 10 ul of the various dilutions of test chemical and solvent are added. Ten (10) ul of solvent are added to the solvent control flasks. Otherwise the test should be conducted as described for readily-soluble chemicals.

(iv) Test procedures dependent on volatility. When volatile chemicals are tested, the following modification of the procedures in Sec(4)(iii)(A) and (B) is recommended. The test is performed as described above (depending on the chemical's solubility in water) except that 250 ml conical

flasks with ground glass stoppers should be used. These flasks are filled as described above with the algal suspension and test chemical solution. For each measurement interval the complete contents of the flasks should be used. Therefore, it is necessary to use a sufficient number of flasks to allow for this destructive sampling with time.

(5) Test results. (i) The results of the measurements should be tabulated. Growth curves resulting from the experiment should be drawn on semi-logarithmic paper (Figure 1).

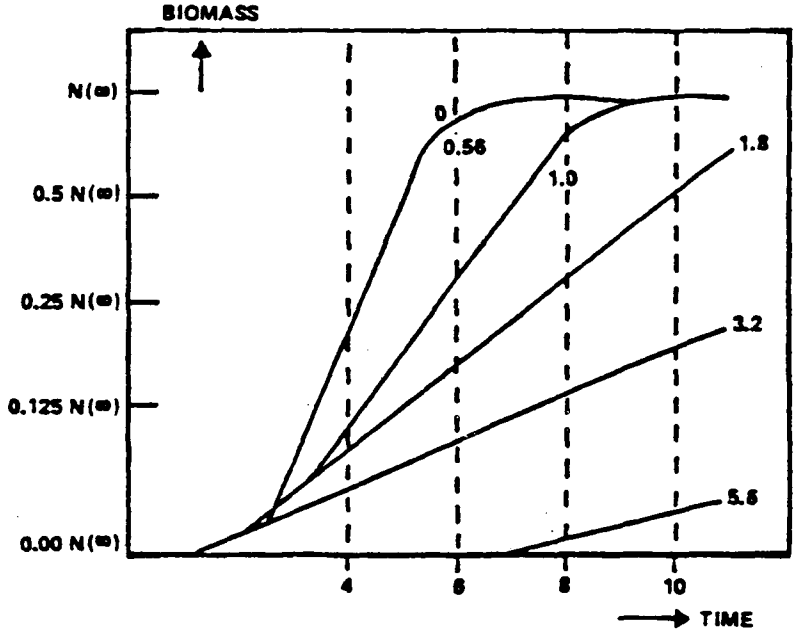

Figure 1. Theoretical example of a result of a toxicity test with algae. The growth curve is plotted on a simple logarithmic scale. The figures give the concentrations of the test compound in weight per volume. The broken lines show the measurements. N(∞) is the maximum cell density that can be achieved.

(ii) The reported chemical effects on algae should include both the EC-50 value and the no-effect level (EC-0). A number of methods have been described for determining the EC50 and EC0. Two examples are provided.

(A) EC-50, and EC-0 determined from the specific maximum growth rate (u). The mean value of the three replicate measurements and the 95 percent confidence limits should be calculated for the 24, 48, 72, and 96 hour sampling times, and plotted as in Figure 1. Two values are selected from the log-linear portion of the curve and the specific growth rate (u) is calculated according to the following formula:

$$u = \frac{\log_{10} \frac{N_1}{N_2}}{0.434\ (t_2 - t_1)}$$

where:

N_1 = the lower cell number chosen in the log phase at t_1

N_2 = the higher cell number chosen in the log phase at t_2

t = time in hours

u = maximum specific growth rate

0.434 = coefficient to convert $\log_{10}$ data to $\log_e$

The percent reduction of the growth rate compared to the control can be calculated:

$$\text{"\% inhibition"} = \frac{1 - u(tox)}{u(b)} \times 100$$

where:

u(tox) = the growth rate in the presence of the chemical

u(b) = the growth rate in the control

The calculated percentages ("% inhibition") are plotted against the log concentration as shown in Figure 2.

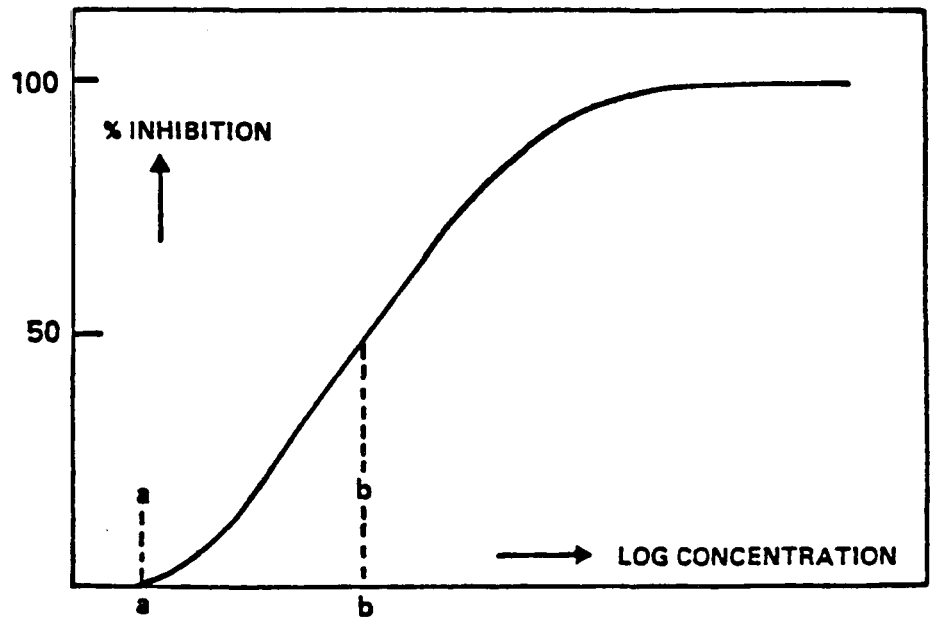

Figure 2. Theoretical example of the relation between the logarithm of the concentration of the chemical and the percentage inhibition.

The highest concentration showing no difference from the control (a) and the EC50 (b) can then be read from this graph.

(B) EC-50 determined from the mean relative growth rate. (1) The mean relative growth rate (RGR) during log phase growth for each culture can be calculated as follows:

$$RGR = \frac{\log_{10} n_2 - \log_{10} n_1}{t_2 - t_1}$$

where:

n_1 = number of cells/ml at t_1

n_2 = number of cells/ml at t_2

t = time (hours)

(2) The RGR of the three control replicates can be calculated and its 95 percent confidence limits determined. Treatment RGR values that are greater than the control upper 95 percent confidence limit indicate algal stimulation and should be ignored. Those above the control RGR but within the 95 percent confidence interval should be assumed to equal the control mean. Treatment RGR values that are less than the lower 95 percent confidence limit of the control should be used in the following calculations: The low treatment RGR values should be expressed as a percentage of the control RGR. The percentage values

including the highest concentration of test compound at which growth is 100 percent that of control (zero inhibition and the EC-0) and the lowest concentration with a value of 0 percent (100 percent inhibition) should be included in an appropriate statistical analysis to determine the EC-50 value and its 95 percent confidence limits.

(3) Interpretation of results. Algal populations rapidly regenerate themselves upon removal of stress; consequently, concentrations that produce effects need careful interpretation.

(6) [Reserved]

(d) Test conditions--(1) Test Species--(i) Selection (A) It is recommended that the algae used be a fast-growing species that is convenient for culturing and testing. The following freshwater species are considered suitable:

(1) Selenastrum capricornutum.

(2) Scenedesmus quadricauda.

(3) Chlorella vulgaris.

(B) Axenic cultures are recommended and are highly desirable when testing biodegradable compounds. However, pure monocultures of algae are required.

(ii) Stock culture. (A) The stock cultures are algal cultures that are regularly transferred to fresh medium to act as initial test material. Cultures that are not used regularly should be streaked out on sloped agar tubes. The tube cultures should be transferred to fresh medium at least once every two months.

(B) The stock cultures should be grown in Erlenmeyer flasks containing the appropriate medium (volume about 100 ml). When the algae are incubated at 20°C with continuous illumination, a weekly transfer is recommended. An amount of "old" culture is transferred with sterile pipettes into a flask of fresh medium, for an approximate 100 fold dilution. The growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate at what density the culture should be transferred to new medium. This should be done before the culture reaches the senescent phase.

(iii) Selection of test algae. (A) Algae used in a test should be in an exponential growth phase, with a cell density of at least 10^4 cells/ml. If it is not possible to use the stock culture directly for testing (due to lack of or excessive cell growth) it may be necessary to pre-culture the algae prior to use.

(B) To pre-culture the algae, 2 liters of sterile nutrient solution are added to a 3 liter Erlenmeyer flask. Sufficient algal suspension from the stock culture is added to give an algal concentration of approximately 10^4 cells/ml ($\pm$ 25 percent). The flasks should be continuously illuminated with fluorescent light, and the temperature maintained at 20°C. Cultures should be shaken by hand at least once every day; this is particularly important for non-motile species. The cell concentration in the culture should be determined daily so that the desired concentration may be obtained for testing.

(C) Other methods of culturing may also be used. Some algae can be grown rapidly in shake cultures. Bubbling with air containing additional carbon dioxide may also accelerate growth. Furthermore, under the culturing conditions described above, the pre-culture,for the algal species recommended in this test, should be optimal for inoculation of test flasks after four to five days. If the algal cultures contain deformed or otherwise abnormal cells (e.g. clumped, chlorotic), they should not be used for the test.

(2) Facilities--(i) Apparatus. (A) In order to avoid contamination with bacteria and other algae, all stock culture maintenance operations should be carried out under

sterile conditions. Possible contamination should be checked by suitable methods.

(B) Testing and culturing of algae should be done in an environmentally controlled cabinet or chamber capable of maintaining a temperature of 20°C $\pm$ 2°C and continuous illumination of approximately 300 to 400 uE/m^2 sec.

(C) In addition to normal laboratory apparatus and equipment for algal testing, a counting apparatus (e.g. Coulter Counter, counting chamber, fluorometer, spectrophotometer, colorimeter) to determine cell numbers is also necessary.

(D) The following apparatus and equipment are necessary for algal culturing:

(1) Incubators or climate rooms capable of maintaining temperature and light at the recommended levels.

(2) Filtering apparatus, accompanying membrane filters, (0.45 um) and 5 liter flask.

(3) Inoculation needle.

(4) Sterile graduated pipettes.

(5) pH-meter.

(6) Culture tubes (150 x 18 mm) with sponge and/or metal caps.

(7) Erlenmeyer flasks, 200 ml, with ground glass stoppers, and a 3 liter flask.

(ii) Dilution water. Redistilled water should be used for preparation of stock, chemical, and nutrient media solutions.

(3) Test parameters--(i) Carriers. If solvents, are used, they should not themselves be toxic at the concentrations used and should not affect algal growth. In no case should the concentration of an organic solvent exceed 0.1 ml/l in the highest concentration.

(ii) Lighting. Algae should be kept under continuous, uniform illumination of approximately 300 to 400 uE/m^2 sec. The light source should be fluorescent lights.

(iii) Loading. For readily water-soluble and volatile chemicals, an algal suspension containing approximately 2 x 10^4 cells/ml should be used. For chemicals with limited water solubility the algal density should be approximately 10^4 cells/ml.

(iv) Temperature. The temperature for culturing and testing algae should be 20°C $\pm$ 2°C.

(v) Nutrient media. The recommended media for adequate growth of algae is shown in Table 1.

TABLE I MEDIUM FOR FRESHWATER ALGAE

Nutrient salts	Amount	
$NaNO_3$	0.5	g
NH_4NO_3	0.33	g
$K_2HPO_4.3H_2O$	0.052	g
$CaCl_2.2H_2O$	0.035	g
$Na_2CO_3.10H_2O$	0.054	g
Fe_3-citrate	0.006	g
Citric acid monohydrate	0.006	g
Trace element solution (see below)	1.0	ml
Redistilled water made up to	1.0	liter
pH	7.7 $\pm$ 0.3	

Trace Element Solution:		
H_3BO_3	2.90	g
$MnCl_2.4H_2O$	1.81	g
$ZnCl_2$	0.11	g
$CuSO_4.5H_2O$	0.08	g
$(NH_4)_6Mo_7.4H_2O$	0.018	g
Redistilled water made up to	1.0	liter

All nutrient solutions can be prepared as concentrated stock solutions using the salts shown in Table 1 and stored in the dark and cold. These solutions should be sterilized by filtration or by autoclaving. The medium is prepared by adding the correct amount of stock solutions, or the nutrients salts directly, to sterile distilled water, to give the final concentrations listed. For solid medium, 1.5 to 2 percent agar can be added. Other media may be necessary if species other than those above are used.

(e) Reporting. In addition to a description of the type of test and method, the report submitted to EPA should include the following information.

(1) For the chemical tested: Manufacturer, empirical formula, batch number and its degree of purity, chemical characterization, (e.g. trade name), and physical properties.

(2) For the test organisms: Origin of innoculum, laboratory culture and strain number, and method of cultivation (including whether cultures aerated and/or shaken).

(3) For the test conditions: Date of beginning and end of the test and its duration, temperature, light intensity and light quality in the growth chamber, type of test flask

(and if closed or open), initial pH of test solution, what carrier is used and how much, concentrations of test chemical, and the counting method.

(4) For the results: A tabulation of cell number or biomass per ml for each flask at each sampling period, the plotted time-growth curves for each concentration and the concentration-effect curve, the EC-50 value and the highest concentration showing no statistical growth inhibition (EC-0) and the statistical methods used to calculate them, and other observed effects, e.g. algicidal vs. algistatic effects, clumping or chlorosis of cells.

(5) For the laboratory performing the test: The name of the person responsible for carrying out the test (study director) as well as the name of the person carrying out the test, the name and address of the testing laboratory, and the date and signature of the person responsible for the test.

(982)

EG-20, OECD
August, 1982

FISH ACUTE TOXICITY TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FISH ACUTE TOXICITY TEST

(a) Purpose. This guideline will be used in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") to fish subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub.L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et. seq.). The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792--Good Laboratory Pracice Standards apply to this test guideline. The following definitions also apply:

(1) "Acute toxicity" is the discernible adverse effects induced in an organism within a short period of time (days) of exposure to a chemical. For aquatic animals this usually refers to continuous exposure to the chemical in water for a period of up to four days. The effects (lethal or sub-lethal) occurring may usually be observed within the period of exposure with aquatic organisms.

(2) "Acute lethal toxicity" is the lethal effect produced on an organism within a short period of time of exposure

to a chemical.

(3) "Confidence limits" are the limits within which, at some specified level of probability, the true value of a result lies.

(4) "LC50" is the median lethal concentration, i.e., that concentration of a chemical in air or water killing 50 percent of a test batch of organisms within a particular period of exposure (which should be stated).

(5) "Static test" is a toxicity test with aquatic organisms in which no flow of test solution occurs. (Solutions may remain unchanged throughout the duration of the test).

(6) "Semi-static test" is a test without flow of solution, but with occasional batchwise renewal of test solutions after prolonged periods (e.g., 24 hours).

(7) "Flow-through test" is a toxicity test in which water is renewed constantly in the test chambers, the chemical under test being transported with the water used to renew the test medium.

(8) "Time-response curve" is the curve relating cumulative percentage response of a test batch of organisms, exposed to a single dose or single concentration of a chemical, to a period of exposure.

(9) "Toxicity curve" is the curve produced from toxicity

tests when LC50 values are plotted against duration of exposure. (This term is also used in aquatic toxicology, but in a less precise sense, to describe the curve produced when the median period of survival is plotted against test concentrations.).

(10) "Units" all concentrations are given in weight per volume (e.g., in mg/liter).

(c) Test procedures--(1) Summary of the test. (i) The aqueous solubility and the vapor pressure of the test chemical should be known prior to testing. The structural formula of the test chemical, its purity, stability in water and light, n-octanol/water partition coeffecient, and pK_a value should be known. The results of a biodegradability test and the method of analysis for the quantification of the chemical in water should also be known.

(ii) The fish are exposed to a range of test substance concentrations preferably for a period of up to 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 percent of the fish (LC50) are determined where possible.

(iii) The maximum concentration tested producing no

mortality and the minimum concentration tested producing total mortality should be recorded.

(iv) For chemicals with limited solubility under the test conditions, it may not be possible to determine an LC50.

(2) [Reserved]

(3) Range-finding test. It may be necessary to perform a range-finding test prior to a definitive test. It provides information about the range of concentrations to be used in the definitive test.

(4) Definitive test. (i) Fish should be exposed to at least five concentrations spaced by a constant factor not exceeding 1.8. A control and solvent control, when appropriate, should also be tested.

(ii) Stock solutions of the required strength are prepared by dissolving the appropriate amount of the test substance in the required volume of dilution water. The pH value of the stock solution should be adjusted to the pH value of the dilution water unless there are specific reasons not to do so. The test should be carried out without adjustment of pH if there is evidence of marked change in the pH of the solution, and it is advised that the test be repeated with pH adjustment and the results reported. This pH adjustment should be made in such a way that the stock solution concentration is not changed to any

significant extent and that no chemical reaction or physical precipitation of the test compound is caused. NCl or NaOH should be used to adjust the pH.

(iii) Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to fish. When such auxiliary substances are used, the control fish should be exposed to the same concentration of the auxiliary substance as that used in the highest concentration of the test substance. The concentration of such auxiliaries should not exceed 0.1 ml/l.

(iv) The chosen test concentrations are prepared by dilution of the stock solution.

(v) For test to be valid, the following criteria apply:

(A) If it is observed that the stability or homogeneity of the test substance cannot be maintained, then care should be taken in the interpretation of the results and a note made that these results may not be reproducible.

(B) The mortality in the controls should not exceed 10 percent at the end of the test.

(C) The dissolved oxygen concentration should have been >60 percent of air saturation throughout the test.

(D) There should be evidence that the concentration of the

substance being tested has been satisfactorily maintained (e.g., within 80 percent of the nominal concentration) over the test period.

(5) Test results. (i) The fish are inspected after 24, 48, 72 and 96 hours. Fish are considered dead if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed, and mortalites are recorded. Observations after the first three hours and six hours are desirable.

(ii) Records are kept of visible abnormalities (e.g., loss of equilibrium, swimming behavior, respirtory function, pigmentation, etc.).

(iii) The cumulative percentage mortality for each recommended exposure period should be plotted against concentration on logarithmic-probability paper. A line is then fitted by eye to these points and the concentration corresponding to the 50 percent response point is read off. This is the LC50 for the appropriate exposure period. Median lethal concentrations also can be calculated using standard procedures given in any of the references cited in section (f). Confidence limits (p=0.95) for the calculated LC50 values can be determined using the standard procedures. The LC50 value should be rounded off to two significant figures.

(iv) Where the data obtained are inadequate for the use of

standard methods of calculating the LC50 (because most of the results are for either no deaths or total mortality, where a dilution ratio of 1.8 has been used) then the highest concentration causing no deaths and the lowest concentration producing 100 percent deaths should be used to determine the LC50 (this being taken as being the geometric mean of these two concentrations).

(6) [Reserved]

(d) Test conditions--(1) Test species--(i) Selection. (A) One of several species may be used, the selection being at the discretion of the testing laboratory. It is suggested that the species used be selected on the basis of such important practical criteria as: their ready availability throughout the year, their ease of maintenance, their convenience for testing, and any economic, biological or ecological factors which have bearing. The fish should be in good health and free from any apparent malformation. If other species fulfilling the the above criteria are used, the test method should be adapted in such a way as to provide suitable test conditions.

(B) Examples of fish recommended for testing and their size are given in Table 1.

TABLE 1--RECOMMENDED SPECIES FOR ACUTE TESTING

Recommended species	Recommended total length (cm)
Brachydanio rerio (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	2.0 ± 1.0
Pimephales promelas (Teleostei, Cyprinidae) Fathead minnow	2.0 ± 1.0
Cyprinus carpio (Teleostei, Cyprinidae) (Linne 1758) Common carp	3.0 ± 1.0
Oryzias latipes (Teleostei, Poeciliidae) (Schlegel 1850) Red killifish	2.0 ± 1.0
Poecilis reticulata (Teleostei, Poeciliidae) (Peters 1859) Guppy	2.0 ± 1.0
Lepomis macrochirus (Teleostei, Centrarchidae) (Linnaeus 1758) Bluegill	2.0 ± 1.0
Salmo gairdneri (Teleostei, Salmonidae) (Richardson 1836) Rainbow trout	5.0 ± 1.0

(ii) Collection or acquisition. The fish mentioned above are easy to rear or are widely available throughout the year. They are capable of being bred and cultivated either in fish farms or in the laboratory under disease- and parasite-controlled conditions so that the test animal will be healthy and of known parentage.

(iii) Holding and acclimation. (A) Fish should be held for at least 12 to 15 days before testing. All fish should be maintained in water of the quality to be used in the test for at least seven days before they are used.

(B) Coldwater fish should be held in tanks containing at least 300 l of water while warmwater fish should be held in tanks containing at least 100 l.

(C) The temperature of the holding water should be the same as that used for testing. The dissolved oxygen concentrations should be maintained above 80% of the air saturation value. A 12 to 16 hour photoperiod should be used.

(D) All fish should be fed three times per week or daily until 24 hours before the test is started.

(E) A batch of fish is acceptable for testing if the percentage mortality over the seven day period prior to testing is less than five. If the mortality is between 5 and 10 percent acclimation should continue for seven additional days. If the

mortality is greater than 10 percent, the entire batch of fish should be rejected.

(2) Test facilities--(i) Apparatus. An oxygen meter, equipment for determination of water hardness, adequate appartus for temperature control, test tanks made of chemically inert materials and other normal laboratory equipment are needed.

(ii) Dilution water. (A) Drinking water (dechlorinated if necessary), good quality natural water, or reconstituted water, with a total hardness of between 50 and 250 mg/l (as $CaCO)_3$ and with a pH of 6.0 - 8.5 are preferred.

(B) Reconstituted water should be prepared from deionized water or distilled water with a conductivity $\leq$ 10 Scm^{-1}. One hundred liters of reconstituted water can be prepared by adding 2.5 l of the following solutions to a tank and bringing the solution to volume with deionized water:

11.76g $CaCl_2 \cdot 2H_2O$/l

4.93g $MgSO_4 \cdot 7H_2O$/l

2.59g $NaHCO_3$/l

2.59g KCl/l

The sum of the calcium and magnesium ions in this solution is 2.5 mmol/l. The proportion of Ca:Mg-ions is 4.13 and of Na:K-ions is 10:1. The acid capacity of this solution is 0.8 mmol/l.

(C) The dilution water should be aerated until oxygen

saturation is achieved and then stored for about two days without further aeration before use.

(3) Test parameters. (i) Constant conditions should be maintained as far as possible throughout the test and, if necessary, semi-static or flow-through procedures should be used.

(ii) The preparation and storage of the test material, the holding of the fish, and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors, and gases and in such a way as to avoid cross-contamination. Any disturbances that may change the behaviour of the fish should be avoided.

(iii) The following parameters are important:

(A) Dissolved oxygen. The dissolved oxygen concentrations should be at least 60 percent of the air saturation value.

(B) Light. A 12 to 16 hour photoperiod should be used.

(C) Loading. A maximum loading of 1.0 g/l for static and semi-static tests is recommended; for flow-through systems a higher loading can be acceptable.

(D) Temperature. Test temperatures of 15 $\pm$ 2°C for rainbow trout and 22 $\pm$ 2°C for carp are recommended. The other recommended species should be tested at 23 $\pm$ 2°C. The temperature should be maintained within $\pm$ 1°C of the selected test temperature throughout the test period.

(E) Feeding. The fish should not be fed during the test.

(e) Reporting. (1) The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of toxicity.

(2) In addition to the reporting requirements prescribed in Part 792--Good Laboratory Practice Standards the reported test data should include the following:

(i) Details of the test procedures used (e.g. static, semi-static, flow-through, aerated, etc.).

(ii) Information about the test organism (scientific name, strain, supplier, any pretreatment, etc).

(iii) The concentrations tested.

(iv) The number of fish in each test chamber and the loading rate.

(v) The methods of preparation of stock and test solutions.

(vi) The dissolved oxygen concentrations, pH values, temperature, total hardness of the test solutions measured each 24 hours and any other available information on water quality.

(vii) Any available information on the concentrations of the test chemical in the test solutions.

(viii) The maximum concentration causing no mortality within the period of the test.

(ix) The minimum concentration causing 100 percent mortality

within the period of the test.

(x) The cumulative mortality in each concentration according to the recommended observation times.

(xi) The LC50 values (based on nominal concentrations) at each of the recommended observation times (with 95 percent confidence limits, if possible).

(xii) A graph of the concentration-mortality curve at the end of the test.

(xiii) The statistical procedures used for determining the LC50 values.

(xiv) The mortality of the control animals.

(xv) Any incidents in the course of the test which might have influenced the results.

(xvi) Any abnormal responses of the fish.

(xvii) A statement that the test was carried out in agreement with the prescriptions of the Test Guideline given above (otherwise a description of any deviations occuring).

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EG-21, OECD
August, 1982

FISH BIOCONCENTRATION TEST

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FISH BIOCONCENTRATION TEST

(a) Purpose. This guideline is to be used for assessing the propensity of chemical substances to bioconcentrate in fish. This guideline describes a bioconcentration test procedure for the continuous exposure of fish to a test substance in a flow-through system. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the hazard a chemical may present to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792--Good Laboratory Practice Standards are applicable to this test guideline. The following definitions also apply:

(1) "Bioconcentration" is the increase in concentration of test material in or on test organisms (or specified tissues thereof) relative to the concentration of test material in the ambient water.

(2) "Bioconcentration factor (BCF)" is the ratio of the test substance concentration in the test fish (C_f) to the concentration in the test water (C_w) at steady-state.

(3) "Depuration or clearance or elimination" is the process of losing test material from the test organisms.

(4) "Depuration rate constant (k_2) is the mathematically determined value that is used to define the depuration of test material from previously exposed test animals when placed in untreated dilution water, usually reported in units per hour.

(5) "Steady-state or apparent plateau" is a condition in which the amount of test material being taken up and depurated is equal at a given water concentration.

(6) "Uptake (u)" is the process of sorbing test material into and/or onto the test organisms.

(7) "Uptake phase" is the time during the test when test organisms are being exposed to the test material.

(8) "Uptake rate constant (k_1)" is the mathematically determined value that is used to define the uptake of test material by exposed test organisms, usually reported in units of liters/gram/hour.

(c) Test procedures--(1) Summary of the test. (i) The test compounds' water solubility, n-octanol/water partition coefficient and stability in water (hydrolysis, photolysis and microbial degradation) should be known prior to testing. The 24 and 96 hour LC50's for the fish species

to be used in the study should also be known. These data should be based on test substance concentrations measured during a flow-through acute toxicity test.

(ii) The procedure proposed is applicable to organic chemicals that are not readily degradable in a microbial degradation test, relatively stable in the aquatic environment and soluble in water at <1 mg/l.

(iii) Before any biological experiments are carried out, the analytical method for the particular substance should be tested. It should be shown experimentally on both water and organisms that the recovery as well as the reproducibility are satisfactory. Blank samples (of water, solvents, etc.) should regularly be analyzed to ensure that no contamination occurs. The detection level should be determined and no quantification should be based on signals which are less than 2.5 times the instrument noise. Organisms and water samples should be removed in such a way that no contamination or losses by adsorption occur.

(iv) This Guideline describes a procedure for characterizing the bioconcentration potential of chemicals in aquatic biota. Parameters used to characterize the bioconcentration potential include the uptake rate constant (k_1), the depuration rate constant (k_2), and the steady-state bioconcentration factor, BCF (k_1/k_2).

(v) Each of three separate groups of test organisms of the same species is exposed to a different concentration of the test material in water; 0, x, and 10x mg/l, where x is defined by analytical and toxicological boundaries. The duration of the uptake phase (3 hrs to 30 days) and depuration phase (6 hrs to 60 days) varies according to the time required to reach the desirable percent of steady-state which is roughly estimated before the test starts. During both phases of the test, organisms and water are periodically removed from the test chambers and analyzed for the test material.

(vi) The uptake rate constant, depuration rate contant(s), bioconcentration factor, and their confidence limits are calculated from the model that best describes the measured concentrations of test material in the organisms and water at any point in time.

(2) [Reserved]

(3) [Reserved]

(4) Definitive test--(i) Test solution preparation. The test material should be added to the dilution water with minimal use of solvents or other carriers. Several systems adaptable to flow-through tests have been described for saturation of water with relatively insoluble test materials

(Chadwick and Kugemgi 1968, Borthwick et al. 1977, Veith and Comstock 1975). Acetone, dimethylformamide, ethanol, methanol, and triethylene glycol are the solvents recommended for use in preparing stock solutions. The concentration of solvent in any test solution should not exceed 0.1 ml/liter in flow-through tests.

(ii) Exposure concentrations. Test fish should be exposed to two or more concentrations of test material in water under flow-through conditions. As a guidance, the highest concentration should be less than one-tenth of the threshold or incipient LC50 for the test species and at least 10 times higher than the detection limit in water and, if possible, each exposure concentration should differ from another by a factor of ten.

(iii) Test duration--(A) Estimation of the uptake phase. (1) As a guideline, the statistically optimum duration of the uptake phase (u) is near the midpoint of an uptake curve plotted on semi-log paper, or $u = 1.6/k_2$, but not more than $3.0/k_2$, which is equivalent to 95 percent of steady-state (Reilly et al. 1977). A pre-test estimate of k_2 may be obtained from:

(i) A test with the same compound and a different species.

(ii) A test with a similar material.

(iii) The results of a preliminary range-finding test.

(iii) Water solubility data.

$$\log k_2 = 0.43 \log(s) - 2.11$$

where (s) is the aqueous solubility in ppm.

(iv) n-octanol/water partition coefficient data,

$$\log k_2 = -0.414 \log (P_{ow}) + 0.122$$

where (P_{ow}) is the n-octanol/water partition coefficient.

(2) The duration of the uptake phase (u) for a test material with log P_{ow} = 3 would be:

$$\log k_2 = 0.414\ (3) + 0.122 = -1.12$$

$$\ldots\ldots k_2 = 0.0759$$

$$u = 1.6/0.0759 = 21 \text{ hours}$$

Similarly, for a test material having a log K_{ow} = 6, the duration of the uptake phase (u) would be:

$$\log k_2 = -0.414\ (6) + 0.122 = -2.362$$

$$k_2 = 0.0043$$

$$u = 1.6/0.0043 = 372 \text{ hours (16 days)}$$

(B) Estimation of the depuration phase. Two times u is usually sufficient time for about 95 percent removal of the body burden ($t_{1/2} = 0.69/k_2$), but several biological or analytical factors may suggest equally acceptable

guidelines. Some compounds follow more complex uptake/depuration behavior than a simple two compartment (C_w and C_f), two parameter (k_1 and k_2) model. For these compounds, longer depuration periods are advisable. On the other hand, the depuration time will most likely be restricted by the lower limit of analytical detection for fish.

(iv) Sampling schedule. (A) As a guideline, no fewer than four uptake sampling times and five depuration sampling times should be spaced throughout the duration of the experiment according to the following fractions of the total time (T_t): first at 0.0278 T_t, second at 0.0556 T_t, third at 0.1111 T_t, fourth at 0.2222 t_t, fifth at 0.3333 T_t (this is the optimum change-over time), sixth at 0.5000 T_t, seventh at 0.6667 T_t, eighth at 0.8333 T_t, and ninth at 1.000 T_t. Table I contains examples of acceptable sampling schedules for bioconcentration tests with test materials with a log P_{ow} = 3.0 and Log P_{ow} = 6.0.

TABLE I--ACCEPTABLE SAMPLING SCHEDULES FOR BIOCONCENTRATION TESTS

Test Phase	Action	Sampling Times log Pow = 3 Hours	Sampling Times Log Pow = 6 Days	No. water samples	No. fish samples
	Start test				
		-1	-2	1	
	Add fish	0	-1	2	
		0.5	0	2	
		1.0	0.5	2	
		1.5	1.0	2	
Uptake		2	1.4	2	4
		4	2.8	2	4
		7	5.7	2	4
		13	11	2	4
		20	17	2	6
	Transfer fish to untreated water	24	20	2	
		30	26	2	4
Depuration		40	34	2	4
		50	43	2	4
Terminate test		60	51	2	6
				28	40

[1] Samples taken after a minimum of 3 tank volumes have been delivered.

(v) Sampling procedures. (A) It is advisable to analyze both water and organism samples as soon as possible after they have been collected to prevent degradation or loss of test material and to determine approximate uptake and depuration rate constants as the test proceeds. If samples cannot be analyzed immediately, it is sometimes appropriate to extract the test material into a solvent, rendering it inert or easier to store until it can be analyzed.

(B) Water samples should be obtained by siphoning through glass tubing from the most central point in the test tank. The sample vessel and siphon should be rinsed with the test solution before collecting the sample.

(C) Water samples are best collected directly into glass vessels of appropriate volume from which the test material can be extracted or analyzed. These vessels might include separatory funnels in the case of organic compounds, or scintillation vials for radioactive test materials.

(D) If significant amounts of particulate matter are present in the water sampled, a second sample should be taken and analyzed after centrifuging to determine whether test material was adsorbed on the particulate matter rather than dissolved.

(E) Water samples containing highly persistent test materials can be stored frozen in plastic containers for later analysis. Care should be exercised to avoid use of containers which could sorb or contaminate samples. With most organic test materials, and especially those tending to degrade easily, a better practice is to extract them from the water and store them under refrigeration in solvent in tightly sealed glass vials.

(F) When removing test organisms for analysis, they should be netted or trapped in a random manner with as little disturbance as possible. If two or more test material concentrations are present, separate nets should be used for each concentration. Organisms should be rinsed with dilution water if accompanied by extraneous matter, blotted dry, and killed by pithing the brain with a dissecting needle or by severing the spinal cord above the opercular region with scissors. They should then be individually weighed and a record made to permit association of the weight with the sample.

(G) Fish may be analyzed as whole fish or as portions, e.g., edible portion (muscle), viscera, remaining carcass, etc. Specific organs may also be analyzed if sufficient biomass is available. If results based on body portions are desired, after the fish is killed it should be eviscerated, taking care not to puncture any parts of the visceral portion which could leak body fluids and possibly cause contamination of the remaining portions. The edible portion or muscle may be removed with a scalpel, blotted dry, and weighed before storing or analysis. The remaining carcass should be weighed before being stored or analyzed. It is necessary to record data for each portion for each

individual fish so that whole body data can be reconstructed based on the sum of the parts.

(H) After weighing, the sample is usually ground or homogenized to promote extraction of test material or to enhance solution of the tissue. Procedures for grinding, extraction, separation of impurities, determination of lipid content, etc., are described in the U.S. Food and Drug Administration's Pesticide Analytical Manual (1975) or the U.S. Environmental Protection Agency's Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Environmental Samples (1974).

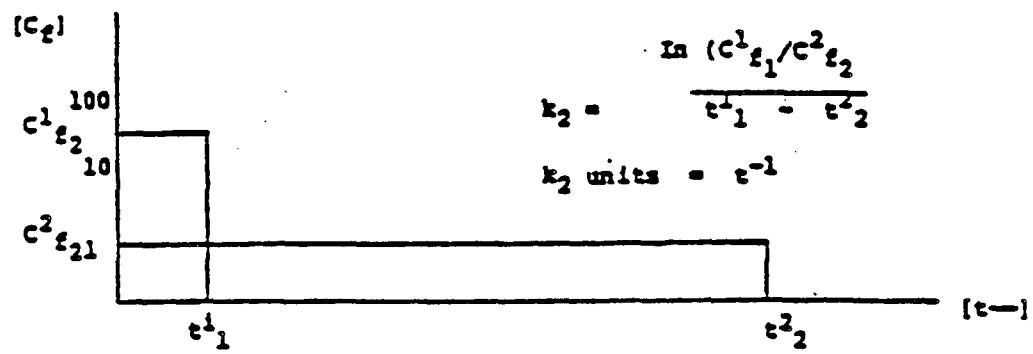
(I) When determining the bioconcentration of test materials which concentrate in lipids, it is often desirable to determine the percent of the total tissue weight made up by lipids. Results between samples are frequently less variable when based on lipid weight rather than on total weight (Reinert 1970).

(J) Organism samples can be wrapped in acetone-rinsed foil, placed in glass jars and frozen if they are not to be analyzed immediately.

(5) Test results. (i) Most bioconcentration data can reasonably be described with a simple two-compartment/two-parameter model as shown by a straight line depuration

profile plotted on semi-log paper. If the depuration profile does not appear to be a straight line, then more complex models can be employed (Blau et al. 1975). Typical variations from the simple model include a third parameter to describe the rate of metabolism of the parent compound or two additional parameters to describe redistribution of the parent compound within the body of the fish. If the best model is in question, it may be worthwhile to estimate parameters for the models in question and to compare the likelihood index of each model according to statistical tests (Blau et al. 1975).

(ii) Graph paper method for depuration rate constant. Plot each concentration of the test material found in fish at each sampling time on semi-log paper. The slope of that line is K_2:



$$k_2 = \frac{\ln (C^1_{f_1}/C^2_{f_2})}{t^1_1 - t^2_2}$$

k_2 units = t^{-1}

(iii) Graph paper method for uptake rate constant. Given k_2, calculate k_1 as follows:

$$k_1 = \frac{C_f \, k_2}{C_w \, (1-e^{-k_2 t})}$$

The value of C_f is read from the smooth uptake/depuration curve near the uptake mid-point on semi-log paper.

(iv) Computer method for calculating uptake and depuration rate constant. The preferred means for obtaining the bioconcentration factor and k_1 and k_2 rate constants is to use nonlinear parameter estimation methods on a digital computer. Two such programs are BIOFAC (Dow) and NONLIN (Proctor and Gamble). These programs find values for k_1 and k_2 given a set of sequential time concentration data and the model:

$$C_f = \begin{cases} C_w \cdot \frac{k_1}{k_2} \left(1-e^{-k_2 t}\right) & 0 \leq t \leq t_c \\ C_w \cdot \frac{k_1}{k_2} \left[e^{-k_2 (t-t_c)} - e^{-k_2 t_c}\right] & t_c \leq t \end{cases}$$

This approach provides standard deviation estimates of k_1 and k_2, and BIOFAC statistically weights the analytical and biological variation of the fish concentration data. These and other non-linear parameter estimation programs are readily available for most computers accepting the Fortran IV language or can be made available from a time-sharing service bureau; they are currently being used by many bioconcentration testing laboratories.

(v) Validity of the test results. (A) Scientific judgement rather than rigid criteria should be exercised in accepting or rejecting bioconcentration test results.

(B) Calculated BCF values based on an octanol/water partition coefficient have a very wide confidence margin (greater than ± 100 percent), but the quality of the value may be better (narrower confidence margin) than an experimental value from a poorly designed study. Generally, the confidence margins for well designed studies approach ± 20 percent. Acceptable bioconcentration data should be reported with confidence margins.

(C) Other criteria for judging the quality of bioconcentration data include the following guidelines:

(1) Percent mortality or adverse effect in control or treated organisms (suggested guideline, 10 percent).

(2) Percent effect of dose on uptake/depuration rate constants (suggested guideline 20 percent).

(3) Percent variation in C_w (suggested guideline, 20 percent) except for the initial dip that may approach 50 percent during the first few days of exposure.

(4) Temperature and dissolved oxygen should not vary more than $\pm$ 1°C and $\pm$ 3 mg/liter.

(5) The importance of actually visualizing an apparent plateau has been a subject of recent debate. It is suggested that 80 per-cent of steady-state (k_1/k_2) in any tissue with a confidence margin of $\pm$ 20 percent is more than sufficient to estimate high quality rate constants for compounds with BCF <10,000. For compounds with BCF >10,000 it may be desirable and acceptable to terminate the uptake phase after a few days not to exceed 28 days even though < 80 percent of steady-state was reached.

(6) A clearly defined uptake/depuration profile is an indicator of high quality bioconcentration data.

(6) Analytical measurements (i) Prior to analyzing fish or water for the test substance, control samples should be spiked with several different concentrations of the test substance and then analyzed. Final values of C_w and C_f should be corrected for recoveries and background.

(ii) Analytical detection limits of test substance in both fish and water should be determined before the bioconcentration test begins and should be documented in the protocol. As a guideline, the limit of detection may be defined as a signal 2.5 times higher than the background noise level.

(iii) If possible, results reported as "not detected at the limit of detection" should be minimized by pre-test method development and experimental design. These results cannot be used for rate constant calculations. The units C_w and C_f should both be expressed either as ppm or ppb.

(d) Test conditions--Test species--(1) Selection.

(i) The procedures regarding selection of which species to test, their source, handling, holding, disease treatment, acclimation, and quality assurance prior to and during testing should be those given in Committee on Methods for Toxicity Tests with Aquatic Organisms (1975).

(ii) The freshwater fish species used most frequently in bioconcentration tests have been rainbow trout, bluegill, and fathead minnows. The most commonly used marine fish have been spot, sheepshead minnows, silversides, shiner perch, English sole, staghorn sculpin and 3-spine sticklebacks. These species are more readily available than most others and can be obtained in convenient sizes.

(2) Facilities--(i) Construction materials. Construction materials and commercially purchased equipment that may contact any water into which test organisms are placed should not contain any substances that can be leached or dissolved by the water. Glass, #316 stainless steel, and perfluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Some will be more suitable than others for use with specific test materials. Unplasticized plastics, cast iron, and concrete can be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, galvanized metal and lead should not come into contact with dilution water, stock solutions, effluent samples or test solutions.

(ii) Toxicant delivery system. (A) One of several toxicant delivery systems can be used successfully, including the proportional diluter (Lemke et al. 1977). Diluters are accurate over extended periods of time, are relatively trouble-free, and have fail-safe provisions. However, proportional diluters often require that laboratories have more than 8 feet of headroom. A small chamber to promote mixing of test material-bearing and dilution water should be used between the diluter and test chambers for each concentration. Design alterations, such

as modification to deliver duplicates of only two or three concentrations, are easy to make (Jarvinen et al. 1977).

(B) Pump systems are relatively simple to understand and use, require little space, and can be quite accurate. Some investigators have found metering pumps to maintain less variable test concentrations than piston operated pumps.

(C) The performance of the toxicant delivery system should be checked before and during each test. This should include determination of the flow rate through each test chamber and measurement of either the concentration of toxicant in each test chamber or the volumes delivered by each portion of the delivery system. The general operation of the toxicant delivery system should be checked daily during the test.

(D) The flow rate through the test chambers should be at least five volume additions per 24 hours, but should take into account the size of the test chamber, the size of the test organisms and the loading. It is usually desirable to construct the metering system so that it can provide at least ten volume additions per 24 hours. The flow rates through the test chambers should not vary by more than 20 percent from any one test chamber to any other or from one time to another within a test.

(iii) Test chambers. Each of the height and width dimensions of the test chamber should be at least 1.5 times the largest horizontal dimension of the test organism. A minimum volume of one liter per fish is crowded but satisfactory for fish up to 15 g; large volumes to fish weight ratios are preferred to minimize the initial C_w dip and to help maintain the dissolved oxygen concentration.

(iv) Cleaning. Metering systems, test chambers, and equipment used to prepare and store dilution water, stock solutions, and test solutions should be cleaned before use. New equipment should be washed with detergent and rinsed with water, pesticide-free acetone, water, acid (such as 5 percent concentrated nitric acid), and twice with tap or other clean water. At the end of every test, all items that are to be used again should be immediately emptied, rinsed with water, cleaned by a procedure appropriate for removing the test material (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic compounds), and rinsed twice with tap or other clean water. Acid is useful for removing mineral deposits, and 200 mg of hypochlorite/liter is useful for removing organic matter and for disinfection. A solution containing 200 mg hypochlorite per liter is conveniently

prepared by adding 6 ml of liquid household chlorine bleach to 1 liter of water. However, acid and hypochlorite should not be mixed because hazardous fumes may be produced. Metering systems and test chambers should be rinsed with dilution water just before use.

(v) Dilution water--(A) General requirements. (1) An adequate supply of dilution water that is acceptable to the test organisms and to the purpose of the test should be available. A minimum criterion for an acceptable dilution water is that healthy test organisms will survive in it for the duration of acclimation and testing without showing signs of stress, such as discoloration or unusual behavior. A better criterion for an acceptable freshwater dilution water is that test organisms will survive, grow, and reproduce satisfactorily in it.

(2) If the dilution water is or is prepared from dechlorinated water, it should be shown that in fresh samples of the dilution water either (i) the concentration of residual chlorine is less than 3 mg/liter or (ii) Acartia tonsa, mysid shrimp, oyster larvae, or first instar daphnids can survive for 48 hours without food. The dilution water should be assayed for the selected test material.

(B) Freshwater. Because daphnids are more sensitive to many toxicants than most other readily available freshwater aquatic animals, water in which first instar daphnids will survive for 48 hours without food is probably acceptable for most short-term tests with freshwater animals. Water in which daphnids will survive, grow, and reproduce satisfactorily should be an acceptable dilution water for longer tests with freshwater animals.

(C) Estuarine and marine water. Because Acartia tonsa, mysid shrimp, and oyster larvae are more sensitive to many toxicants than most other estuarine and marine aquatic animals, water in which they will survive for 48 hours without food is probably acceptable for most short-term tests with estuarine and marine animals. Water in which Acartia tonsa or mysid shrimp will survive, grow, and reproduce satisfactorily should be an acceptable dilution water for longer tests with estuarine and marine animals.

(e) Reporting. In addition to the reporting requirements prescribed in Part 792--Good Laboratory Practice Standards, the test report should include the following information:

(1) A detailed description of the test material, including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and identity and concentration of any carriers (solvents) or other additives used;

(2) The source of the dilution water, its chemical characteristics, and a description of any pre-treatment;

(3) Detailed information about the test organisms, including scientific name and how verified (and strain for salmonids when appropriate), weight (wet, blotted dry), standard length of fish, height of bivalve molluscs, age, life stage, source, history, observed diseases, treatments, acclimation procedure, and food used;

(4) A description of the experimental design and metering system;

(5) Description of tissue and water samples analyzed, and methods used to obtain, prepare, and store them;

(6) Methods used for, and results (with standard deviation) of all chemical analyses of water quality and concentration of test material in tissue and water, including validation studies and reagent blanks;

(7) The steady-state bioconcentration factor, the uptake and depuration rate constants, the confidence margins (± standard deviation) and the method of computations/data analysis;

(8) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

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50272-101

REPORT DOCUMENTATION PAGE	1. REPORT NO. EPA 560/6-82-002 Part 2	2.	3. Recipient's Accession No. PB82-232992
4. Title and Subtitle Environmental Effects Test Guidelines			5. Report Date August, 1982 6.
7. Author(s)			8. Performing Organization Rept. No.
9. Performing Organization Name and Address Office of Pesticides and Toxic Substances Office of Toxic Substances (TS-792) United States Environmental Protection Agency 401 M Street, S.W. Washington, D.C. 20460			10. Project/Task/Work Unit No. 11. Contract(C) or Grant(G) No. (C) (G)
12. Sponsoring Organization Name and Address			13. Type of Report & Period Covered Annual Report 14.

15. Supplementary Notes

16. Abstract (Limit: 200 words)

These documents consitute a set of 21 environmental effects test guidelines (and, in some cases, support documents) that may be cited as methodologies to be used in chemical specific test rules promulgated under Section 4(a) of the Toxic Substances Control Act (TSCA). These guidelines cover testing for invertebrate toxicity, aquatic vertebrate toxicity, avian toxicity, phytotoxicity, and bioconcentration. The guidelines will be published in loose leaf form and updates will be made available as changes are dictated by experience and/or advances in the state-of-the-art.

17. Document Analysis a. Descriptors

b. Identifiers/Open-Ended Terms

c. COSATI Field/Group

18. Availability Statement	19. Security Class (This Report)	21. No. of Pages
Release unlimited	Unclassified	532
	20. Security Class (This Page)	22. Price
	Unclassified	

(See ANSI-Z39.18) See Instructions on Reverse

OPTIONAL FORM 272 (4-77)
(Formerly NTIS-35)
Department of Commerce